HISTOCHEMICAL REACTIONS OF ADRENAL CORTICAL CELLS [1]

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INTRODUCTION

The purpose of this paper is to report observations on some histochemical reactions of cortical cells of fresh and fixed adrenal glands. Varying interpretations of the functional activity of adrenal glands have been based upon the cytological appearances and reactions of cortical cells to histochemical tests. One group of investigators has maintained that the ketosteroid content of the adrenals can be visualized (Deane and Greep, '46; Dempsey and Wislocki, '46; Dempsey, '48; Greep and Deane, '49). By employing a battery of procedures, chemical and physical, they have contended that adrenal ketosteroids can be characterized in a semi-quantitative manner. Other investigators have denied these claims and have asserted that at the present time cortical steroids cannot be found by morphological examination (Albert and Leblond, '46; Boscott and Mandl, '49; Claesson and Hillarp, '47). Few have determined the role of formaldehyde fixation in effecting positive results (Yoffey and Baxter, '47) nor have the histochemical procedures with respect to fixation been evaluated.

MATERIAL AND METHODS

Adrenals of Sprague-Dawley male rats were used in all the experiments. The glands were sectioned on the freezing microtome 13–16 μ in thickness. Sections were prepared from: (1) fresh adrenals within 30 minutes of the animal's death;

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(2) unfixed adrenals kept in homologous serum in the refrigerator for 72 hours; (3) adrenals fixed in 10% buffered formaldehyde, pH 7.2, for two hours, for 48 hours, and for three or more months. Tissue fixed in formaldehyde was washed in running tap water for 15 hours before being used. Each histochemical test was performed on the different fresh and fixed sections at the same time and with the same reagents.

The following tests and procedures were employed.

I. 1. 2–3 naphthoic acid hydrazide diazo blue B reaction, hereafter to be called NAHD, according to the directions of Ashbel and Seligman ('49).

2. Reduced fuchsin, prepared according to Lison ('36), but using "new fuchsin" C. I. no. 678 (Conn, '46). Sections were placed in this reagent for 10–12 minutes and then washed in three changes of sulfurous acid prepared by mixing equal volumes of 10% potassium metabisulfite and 1 N HCl and diluting with 10 volumes of distilled water.

Both the NAHD and reduced fuchsin reactions were always employed in parallel procedures. With these reagents a variety of methods were used on the sections. Tissues were handled in the following way: (1) they were placed directly into NAHD and leukofuchsin; (2) they were exposed to N/10 mercuric chloride for 5 minutes at room temperature and then treated with the two reagents; (3) they were immersed in N/10 HCl for 15 minutes at room temperature and then treated with NAHD and leukofuchsin; (4) in some instances hydroxylamine was employed as a blocking agent before and after exposure of sections to N/10 HCl and N/10 mercuric chloride as suggested by Danielli ('49) and the tissues were reacted with NAHD and leukofuchsin.

All sections were washed in water for 5 minutes after treatment with any reagent except leukofuchsin. Following immersion in leukofuchsin tissues were passed directly into sulfurous acid wash.

II. Sudan staining. Both Sudan IV and Sudan black B were employed. Sudan IV was prepared as a saturated solution in equal parts of acetone and 70% alcohol and as a satu-

rated solution in a mixture of 50 cm^3 of equal parts of acetone and 70% alcohol and 75 cm^3 of absolute ethylene glycol (Chiffelle and Putt). Sudan black B was used as a saturated solution in 70% alcohol and as a saturated solution in absolute ethylene glycol (Chiffelle and Putt).

III. Polarizing microscopic examination. Unstained and stained preparations were examined under the polarizing microscope to determine the presence of birefringent material and its relation to the sudanophilic droplets.

IV. Acetone solubility. Control sections for all reactions were placed in absolute acetone for one hour at room temperature before applying each of the above procedures.

RESULTS

I. Histochemical reactions

Fresh adrenal tissue

When sections of fresh tissue were treated with reduced fuchsin and NAHD reagents both the droplets and cytoplasm of the cortical cells were unstained. With Sudan IV and Sudan black B droplets throughout the cortex were colored a bright orange red and dark green respectively; the cytoplasm remained colorless. In sections treated with acetone the droplets remained uncolored by the Sudans. A few droplets of the outer fasciculata showed birefringence in the polarizing microscope and this was abolished after treatment with acetone.

When contiguous sections of the same fresh tissue were exposed to mercuric chloride the cytoplasm of cortical cells was colored lavender by reduced fuchsin; pre-treatment with acetone removed some cytoplasmic staining but a very faint pink hue persisted. Droplets were colorless following immersion in leukofuchsin. With NAHD both cytoplasm and droplets remained unstained. Exposure to mercuric chloride did not alter the appearance of sections stained in the sudans or examined under the polarizing microscope.

When sections of fresh tissue were immersed in N/10 HCl the cytoplasm of cortical cells stained a faint lavender with reduced fuchsin which was not removed by acetone treatment. All other reactions were the same as described above following exposure to mercuric chloride.

Unfixed adrenal tissue kept in serum for 72 hours at icebox temperature

Cortical cells exhibited the same reactions to all tests as the fresh tissue.

Adrenal tissue exposed to formaldehyde for two hours

Sections which were placed directly into leukofuchsin without previous exposure to mercuric chloride or N/10 HCl showed a faint lavender color in the cytoplasm which was not removed by acetone. All other reactions of cytoplasm and droplets were the same as for fresh tissue.

Adrenal tissue fixed in formaldehyde for 48 hours

Both reduced fuchsin and NAHD gave identical patterns of stain in cortical cells. When sections were placed directly into these reagents the cytoplasm of the cells of the glomerulosa and outer half of the fasciculata was moderately positive, i.e., stained purple or blue. Previous treatment with acetone abolished the reactions. In the glomerulosa and outer fasciculata a variable number of cells (approximately 25–50%) contained droplets that reacted positively to leukofuchsin and NAHD reagents. The remaining cells contained unstained droplets which, however, could be colored by the Sudans. Positive reactions in droplets were prevented by pre-treatment with acetone. Sections placed in Sudan stains colored droplets of all cells throughout the cortex. Under the polarizing microscope many Sudan positive droplets contained birefringent material, predominantly in the outer cortex, but no birefringent material was seen with certainty in NAHD or

leukofuchsin positive droplets. Treatment with acetone removed all positive reactions in droplets.

The reactions of cytoplasm and droplets to the above procedures after exposure to mercuric chloride and N/10 HCl

TABLE 1

PROCEDURE	CYTOPLASM		DROPLETS	
	Leukofuchsin	NAHD	Leukofuchsin	NAHD
Tissued placed directly in reagents				
Fresh tissue	0	0	0	0
Two hours in form.	Faint +	0	0	0
48 hours in form.	++	++	+ (25–50%)	+ (25–50%)
Three months in form.	+++	+++	0	0
Tissues exposed to mercuric chloride				
Fresh tissue	+	0	0	0
Two hours in form.	+	0	0	0
48 hours in form.	++	++	+ (25–50%)	+ (25–50%)
Three months in form.	+++	+++	0	0
Tissues exposed to N/10 HCl				
Fresh tissue	Faint +	0	0	0
Two hours in form.	Faint +	0	0	0
48 hours in form.	++	++	+ (25–50%)	+ (25–50%)
Three months in form.	+++	+++	0	0
Tissues pretreated with acetone				
Fresh tissue after exposure to $HgCl_2$	Faint +	0	0	0
Two hours in form.	Faint +	0	0	0
48 hours in form.	Faint +	0	0	0
Three months in form.	Faint +	0	0	0

were identical to those described above, both in intensity and distribution.

When sections were immersed in hydroxylamine for one hour at 37°C. all reactions with leukofuchsin and NAHD were negative, with or without previous exposure to mercuric chloride and N/10 HCl.

Adrenal tissue fixed in formaldehyde for three months or more

As before, reduced fuchsin and NAHD gave identical patterns of staining in cortical cells. When treated with these reagents the cytoplasm was strongly positive while practically all of the droplets remained unstained. With the Sudans the droplets of all cells were positive and the cytoplasm remained colorless. Abundant birefringent crystals were found in many, but not all, of the droplets of the outer cortex and occasionally in the glomerulosa and reticularis. Immersion in acetone removed sudanophilic and birefringent material.

Exposure to mercuric chloride and N/10 HCl did not alter the results obtained without such treatment.

Table 1 presents the collected data of these various procedures.

II. Evaluation of NAHD reagent

According to Ashbel and Seligman ('49) the NAHD reagent is specific for ketosteroids, or at least for $R-\overset{C}{\underset{R}{}}=0$ as distinguished from $RHC=0$. In order to evaluate this procedure two series of experiments were run. In the first the reagent was tested against pure aldehydes *in vitro*. In the second it was compared with reduced fuchsin as to parallelism of staining and sensitivity.

A 0.1% solution of 2–3 naphthoic acid hydrazide was prepared in 50% aldehyde-free alcohol. To 50 cm^3 of this solution 1 cm^3 of liquid benzaldehyde was added. The mixture was stirred at irregular intervals for 1½ hours at room temperature. Five volumes of water were then added to the mixture and a white flocculent precipitate appeared which was filtered and washed with water and then dried in a 100°C. oven. A small portion of the dried flaky material was put into 50 cm^3 of equal parts of absolute alcohol and phosphate buffer, pH 7.38. To this was added 50 mg of diazo blue B. The undissolved flakes turned a deep blue in less than one minute.

When the above precipitate was recrystallized from absolute alcohol a dry powder was obtained which was partially soluble

in the alcohol-phosphate buffer solution. When diazo blue B was added the solution turned a deep blue-purple.

Acetaldehyde and formaldehyde gave similar results with diazo blue B, a deep purple color developing after addition of the dye to the hydrazide-aldehyde compound. When diazo blue B was added to naphthoic acid hydrazide alone the color which appeared ranged from yellow to brown; with each of the above aldehydes alone the dye produced varying colors ranging from yellow to orange or red. There was no blue component in the color developed by mixing diazo blue B with napthoic acid hydrazide or with the aldehydes alone.

TABLE 2

	$\frac{N/10}{HCl}$	$HgCl_2$	NH_2OH	$\frac{N/10}{HCl}$	$HgCl_2$	CYTOPLASM					
						Fresh		48 hrs. in form.		3 mos. in form.	
						Schiff	NAHD	Schiff	NAHD	Schiff	NAHD
A						—	—	++	++	+++	+++
B			X			—	—	—	—	—	—
C			X	X		+	—	+	—	+	—
D	X		X			—	—	—	—	—	—
E			X		X	+	—	+	—	+	—
F		X	X			—	—	—	—	—	—
G		X				+	—	++	++	+++	+++

A second series of experiments was run to compare the reactions of cortical cells to reduced fuchsin and NAHD. Adrenal sections, fresh and formaldehyde fixed, were subjected to the following procedures and then stained with the two reagents. The steps of each procedure are presented in sequential order.

A. Sections → reduced fuchsin and NAHD.
B. Sections → hydroxylamine → reduced fuchsin and NAHD.
C. Sections → hydroxylamine → N/10 HCl → reduced fuchsin and NAHD.
D. Sections → N/10 HCl → hydroxylamine → reduced fuchsin and NAHD.

E. Sections → hydroxylamine → mercuric chloride → reduced fuchsin and NAHD.
F. Sections → mercuric chloride → hydroxylamine → reduced fuchsin and NAHD.
G. Sections → mercuric chloride → reduced fuchsin and NAHD.

The full results are presented in table 2. When fresh or fixed sections were immersed in hydroxylamine for one hour and then exposed to N/10 HCl or N/10 mercuric chloride the cytoplasm of cortical cells was faintly positive to reduced fuchsin but negative to NAHD. Further when fresh tissue was treated with mercuric chloride the cytoplasm was colored a faint lavender by reduced fuchsin but was unreactive to NAHD. Otherwise the distribution and the intensity of reaction were the same for both reagents.

DISCUSSION

The following observations were significant in comparing the leukofuchsin and NAHD reagents: (a) fresh unfixed adrenals gave negative reactions with both leukofuchsin and NAHD reagents in both cytoplasm and droplets; (b) fresh unfixed adrenal tissue immersed in mercuric chloride showed cytoplasm, but not droplets, which was positive to reduced fuchsin; (c) fixation in formaldehyde beyond two hours and at least up to 48 hours produced a positive reaction with leukofuchsin and NAHD in cytoplasm and droplets; (d) fixation in formaldehyde for increasing periods of time increased the intensity of cytoplasmic staining with NAHD and reduced fuchsin; (e) fixation of adrenal tissue in formaldehyde for three months or more showed cortical cell droplets which did not react with NAHD or leukofuchsin.

The observations suggest that either free aldehydes are not present in fresh adrenal tissue or that if present their reactivity is so great that they are quickly lost. The action of mercuric chloride upon tissues, as in the plasmal reaction, is unknown, but *in vitro* experiments suggest that it may either

oxidize unsaturated fatty acids to form carbonyl groups or break the acetal linkage between fatty acids and glycerol to form aldehydes (Cain, '49; Danielli, '49; Tannhauser and Schmidt, '46). The system in tissue is undoubtedly more complex than in the test tube. It was noted that tissue kept in serum for 72 hours at icebox temperature and exposed to air over this period gave the same results with leukofuchsin and NAHD as did fresh adrenals. As with fresh tissue, immersion in mercuric chloride was necessary to obtain a positive leukofuchsin reaction. Hence it must be assumed that oxidation of unsaturated fatty acids did not occur with exposure to air or that they are not present in tissues in a state readily oxidized. Whatever the action of mercuric chloride is upon tissue, a positive reaction with leukofuchsin and NAHD merely denotes the presence of carbonyl groups and does not reveal the role of mercuric chloride in effecting this change nor does it reveal the nature of the compound acted upon.

Since the cytoplasm of fresh tissue after exposure to mercuric chloride reacted faintly with reduced fuchsin and since this was not abolished by pre-treatment with acetone, the results indicate the release of carbonyl groups in the cytoplasm, possibly non-lipid.

Of greater importance was the fact that the droplets in fresh cortical cells, exposed to mercuric chloride, were sudanophilic but leukofuchsin and NAHD negative. This strongly implies that carbonyl groups, either as ketones or as aldehydes, were not present in the droplets, in a free or bound state. Boscott and Mandl ('49) and Gomori ('42) reached the same conclusions.

In addition it was noted that not all of the sudanophilic droplets contained biefringent crystals, and that none of the leukofuchsin and NAHD positive droplets contained birefringent material. It has been stated that if a locus within a cell is sudanophilic, birefringent, Schiff plasmal positive, autofluorescent and acetone soluble, that locus contains ketosteroid (Deane and Greep, '46; Dempsey, '48; Greep and Deane, '49). The droplets of cortical cells are claimed to fulfill these 5 re-

quirements. From the data in this paper it is apparent that a positive reaction to these tests is not present in a single locus.

There is further evidence that a battery of 5 histochemical tests is not a reliable indication of the presence of ketosteroids. Claesson and Hillarp ('47) have reported that the birefringent material in the ovary, in tissues and *in vitro,* gave cholesterol reactions but was not identical with the phenylhydrazone forming substance. Boscott et al. ('48) studied the Schiff plasmal reaction on three pure samples of desoxycorticosterone. The hormone was treated with aqueous mercuric chloride. No reaction was obtained with the Schiff's reagent. Ashbel and Seligman ('49) employing the NAHD reagent obtained positive reactions with several pure ketosteroids but were unable to obtain a positive reaction in fresh adrenal tissue. Yoffey and Baxter ('47) reported that birefringent material was not seen in the cortical droplets of fresh tissue. With increasing time doubly refractile crystals appeared in droplets. Following formaldehyde fixation the birefringent material was abundant in the cortex. X-ray powder studies of the crystals failed to establish the identity of the crystals with ketosteroids or cholesterol.

The nature of the tissue alteration produced by formaldehyde fixation is unknown. The actions of formaldehyde on proteins are complex and multiple (Baker, '45; French and Edsall, '45; Karrer, '46). It itself can form polymerization products by hydration and can polymerize some sugars (Karrer, '46). Its reactions with lipids and steroids, either *in vitro* or in tissue, have not been determined.

From the results of this investigation it appears that formaldehyde produced a rearrangement of chemical radicals or bonds in the cell constituents. Prior to formaldehyde fixation neither cytoplasm nor droplets was positive with leukofuchsin or NAHD reagents. Two hours of fixation had no noticeable effect, at least with respect to these two reactions, but 48 hours caused droplets and cytoplasm to become positive with both reduced fuchsin and NAHD, without previous mercuric chloride treatment. This implies that formaldehyde fixation can

free bound aldehydes and ketones, permits new ones to be formed, or cannot itself be completely removed from tissues by washing.

The specificity of NAHD for ketones is still uncertain. Ashbel and Seligman ('49) state that phenylhydrazones of aldehydes couple with diazonium compounds in pyridine to form water-insoluble formazans, while the phenylhydrazones of ketones will not couple because of the absence of replaceable hydrogen. Using these reactions they found no free aldehydes in the adrenal cortex of formaldehyde fixed tissue although a positive result was obtained with NAHD reagent. In their paper, however, no mention was made of the reaction *in vitro* of aldehydes to NAHD. The data of this study demonstrated conclusively that aldehydes *in vitro* form blue compounds with NAHD reagent. Furthermore, the distribution of the stain in adrenal tissue was almost precisely the same as the leukofuchsin stain, and indeed, appeared to be less sensitive.

CONCLUSIONS

Fresh and formaldehyde fixed adrenal tissues were subjected to a variety of histochemical tests. It was found that carbonyl groups are not demonstrable in the cortical cells of fresh adrenals and that positive tests for carbonyl groups appear only after exposure to formaldehyde and mercuric chloride. Droplets of cortical cells react with leukofuchsin and naphthoic acid hydrazide diazo blue B after 48 hours of fixation in formaldehyde. After three months exposure to formaldehyde they no longer react with these reagents. Birefringent crystals are sparsely scattered in the cortex of fresh adrenals but are numerous in formaldehyde fixed tissue.

From this study and the work of others there appears to be no single histochemical test or combination of tests that will visualize ketosteroids.

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AN IMPROVED TANTALUM CHAMBER FOR PROLONGED MICROSCOPIC STUDY OF LIVING CELLS IN MAMMALS[1]

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SIX FIGURES

Since Sandison ('24) first demonstrated the feasibility of installing in an animal a transparent device into which vessels and connective tissue would grow and be thin enough for repeated microscopic study many variations and improvements of the method have been described. The method was conceived by Dr. E. R. Clark and originally developed in his laboratory (Clark et al., '30). Most of the variations were made with some special problem in mind. However, they all had one limitation, namely, lack of permanence. This was no defect if experiments could be completed in one year or less. After that time chambers were extruded or became useless for other reasons. The useful life of a chamber was extended (Williams, '48) by making it of tantalum, glass and mica in such a manner that connective tissue and vessels could grow through the body of the chamber and effectively anchor it in place. For practical purposes these chambers became a part of the animal in the sense that trauma or growth processes or even severe local infections would rarely cause extrusion.

[1] This work was supported by grants from the American Cancer Society and The Cancer Research Fund of the University of Pennsylvania, established by a gift from Anna May Conway and a bequest of Louise M. Holroyd.

In some respects the tantalum chamber as described resembled the glass chamber from which it was derived (Williams, '39). An extended experience with tantalum chambers indicated that they need not incorporate any important features of the older glass ones and, therefore, construction could be simplified in that glass, which is difficult to work, could be eliminated. Simplification of construction cannot be carried too far if permanence and facilities for transplanting are essential to the studies contemplated. In our experience no chamber made of solid pieces of plastic, glass or metal has had a useful life of more than one year and most of them were extruded much sooner than that. The reason for this is that solid, smooth devices to which tissue will not adhere firmly are, when installed, lying loose in a bed of living tissue which has capacity to extrude such foreign bodies if they are partially or completely exposed.

The improved chamber herewith described is the result of numerous experiments to determine the minimum number of parts of which a chamber could be composed and still answer the requirements of transplanting and permanence. Experiments were also made to determine optimum sizes, shapes and sequence of parts so that the devices would not only be standardized for use in rabbit ears but could be used without change in skin flaps made surgically on any of the common laboratory animals as large or larger than a rat (Williams, '34). Many materials and combinations of materials have been used. Some plastics that are easy to fashion are decidedly irritating to living tissues. In the chamber herewith described only tantalum and mica come in contact with living tissue. We have found both of these materials to be non-toxic to living cells when properly cleaned. Over many months capillaries with the endothelial wall in direct contact with either tantalum or mica showed no sticking of leucocytes, a delicate sign of vascular injury.

The parts of a chamber arranged in the order in which they are assembled are illustrated, natural size, in figure 1. All

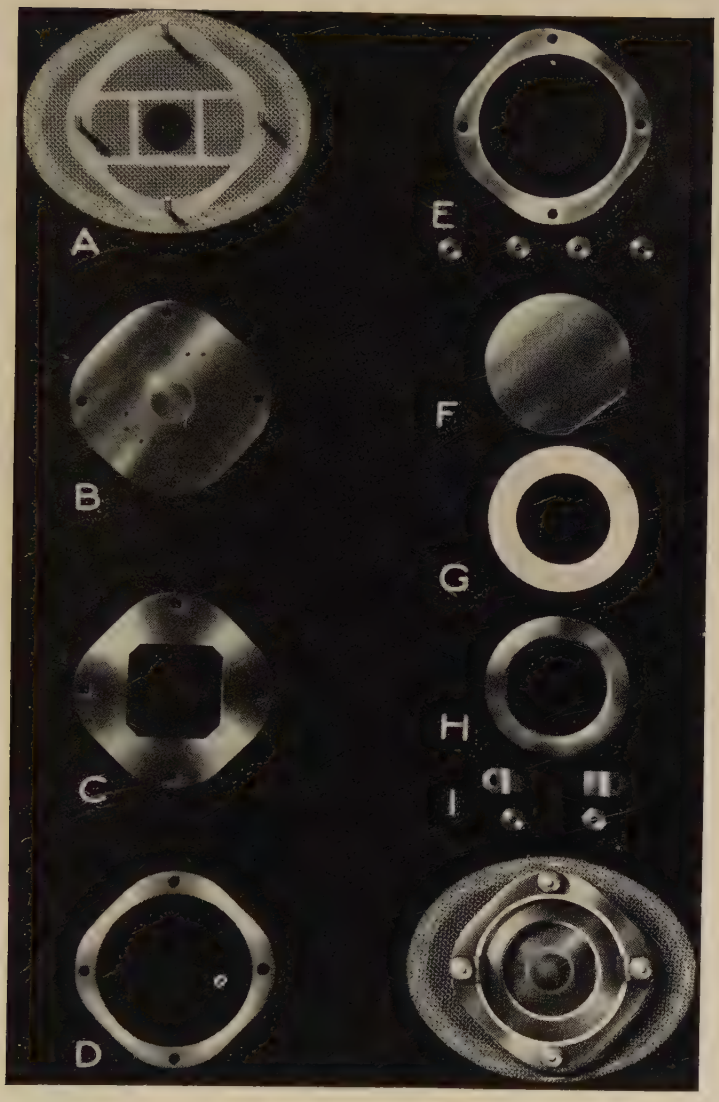

Fig. 1 The parts of a chamber, natural size, arranged in the order in which they are assembled. Descriptions of the various pieces are given in the text.

dimensions, except thickness, may be taken from the photograph.

A is the body of the chamber, the part which holds it in place when installed. It consists of a central piece cut with a jeweler's saw from sheet tantalum .012″ thick and finished with a file.[2, 3] The central bars are not necessary for strength but are essential in the control of epidermis and infection. This part is included between two layers of tantalum wire gauze. The gauze is supplied in only one size of mesh. Using a plastic pattern, a piece of gauze is cut to the external dimension of A. Another larger piece is cut so that it will project in all directions 3 mm beyond the first when the latter is laid on it. Four tantalum bolts, 120 threads to the inch, 5/16″ long and made of .040″ wire, are placed in the holes in the center piece which is then laid on the larger of the two pieces of gauze. The smaller gauze piece is forced down over the bolts and the plastic pattern, which has holes spaced to accommodate the bolts, is laid on it. The projecting edges of the larger piece of gauze are bent over the smaller. When

[2] Thickness of stock and some other dimensions are given in inches because manufacturers and suppliers do not ordinarily describe and price materials according to the metric system.

[3] Tools useful in makng chambers: dental motor equipped with a flexible shaft, hand chuck and an assortment of round dental burrs; jeweler's saw with an assortment of fine blades; dissecting lens on a stand; small vise; drill press and a number of high speed twist drills no. 59–.041″; set of escapement files; micrometer; socket wrench for handling the brass nuts (this tool and the following jig are not available commercially); steel jig for forming heads on tantalum bolts. The design for this jig was kindly sent to us by Dr. G. H. Algire and Frances Y. Legallais. It consists of two small accurately machined steel blocks aligned with guide pins and held together with two bolts and wing nuts. On one side of the jig, holes .040″ in diameter, are drilled in the line where the blocks meet. These holes are 1 mm less in depth than the length of the bolt desired. The projecting portion of the bolt is upset to form a head. It cannot be simply battered down with a hammer without compressing the threads. This compression can be prevented and the head formed by using in succession the following punches from an "Inverto" Staking Set, a very sharp center punch, then punches nos. 18, 117 and 106. Punch no. 52 is then inverted into the staking stand and the head finished with punch no. 33.

the pattern is removed the bending of the gauze is completed and fastened by sewing with .003″ tantalum wire. The center hole is cut out with scissors.

B is a piece of clear mica, 150 μ thick. Mica is split under running water with a needle and the thickness measured with a micrometer. The holes in B are drilled from both sides under a lens using a dental burr in the chuck of a flexible shaft attached to the mandrel of a variable speed motor. The small holes are placed so that they will be just outside the center square of bars in A when B is placed on A. The central ring is cut from a sheet of 1/4″ lucite and attached to the mica with Duco cement. The wall is about 1/2 mm thick. When the chamber is assembled this ring fits in the center hole in A and serves to prevent epidermis from closing the opening through which light is transmitted to the chamber contents.

C is the tantalum foil spacer which regulates the thickness of tissue that grows into the chamber. It is about 18 μ thick. This is the minimum space into which vessels will grow. Such a layer of tissue is too delicate for most transplanting experiments. Consequently, an additional space is used when the chamber is later to be opened. It is made of mica, 25 μ thick, cut to dimensions and shape of piece G. When a chamber is assembled it rests free inside piece D.

D is made of sheet tantalum .012″ thick, the holes being drilled with a .041″ twist drill. Its chief purpose is to give rigidity to E.

E is made of lucite or plexiglass 2 mm thick. When E is placed on D in the assembled chamber a well is created for holding fluid during grafting operations. This piece does not come in contact with living tissue.

F is the mica cover slip, 100 μ thick. It should fit freely in E.

G is a filter paper sealing washer. When the chamber is being installed it is impregnated with 50° paraffin and placed over F before the paraffin solidifies completely.

H is a copper ring for retaining the cover. It is held in place by the phosphor bronze springs, I.[4]

Individual parts of chambers except the springs are made from templates and are interchangeable. They are made a few at a time whenever it is convenient and a stock accumulated.

All tantalum pieces are cleaned and sterilized by immersing successively in carbon tetrachloride, 5% nitric acid and 95% alcohol — 30 minutes in each. Mica parts are cleaned by wiping with alcohol and sterilized by flaming. Other parts are not sterilized.

In purchasing rabbits for long term studies the following specifications are given: they should be about one-half grown, free from disease and ear mites and have broad, thick skinned ears with a minimum length of 5 inches. Colored animals are preferable since in our experience albinos are likely to be more excitable. Whenever possible animals are purchased in litters.

The installation of chambers is done with aseptic precautions. However, it is of greater importance to dissect with a minimum of trauma and to have no tension at any point once the chamber is installed.

Installing a chamber in an ear is a simple procedure requiring about 30 minutes. Although chambers may be placed in both ears, we have done so rarely unless necessary for certain experimental controls. The left ear fits under the microscope most readily and is the one generally used. In preparing an ear for operation it is undesirable to shave it or use a depilatory. All long hair is clipped off and the site of operation clipped somewhat closer with scissors. The ear is washed

[4] Sources from which materials may be obtained: tantalum — Ethicon Suture Laboratories, Division of Johnson and Johnson, New Brunswick, N. J.; clear India black mica — William Brand Co., 276 Fourth Avenue, New York 10, N. Y.; tantalum bolts and brass nuts, the bolts without heads — made on order by Waltham Screw Co., 77 Rumford Avenue, Waltham 54, Mass.; plexiglass — Plastic Center, 2015-17 Market St., Philadelphia, Pa. These sources furnish only stock materials. Finished chamber parts are not available commercially and must at present be made by hand.

with soap and water followed by 95% alcohol and then infiltrated completely around the base with about 4 cm^3 of 2% procaine.

All dissection is done from the inside of the ear. A kodaloid or other plastic pattern, 2 mm larger in all diameters, than piece A, figure 1, if placed against the inside of the ear and disposed so that the central artery is at one side of the center hole in A, and so that the border vein is outside the pattern. Four evenly spaced needles are thrust through the ear at the edge of the pattern and the pattern removed. These needles serve as guides for the dissection that follows. A small incision in the center of the long axis of the marked region is made just through the inside skin. The skin is raised around the incision so that a clear view of the cartilage is obtained. A short incision is made in the cartilage but not through the perichondrium of the opposite side. A blunt dissector is then inserted between the outside of the cartilage and the perichondrium and the dissection extended so that the separation involves the entire region inside the needles. The needles are removed and with scissors the inside skin and attached cartilage are cut from end to end of the long axis. Dissection is now complete and the chamber may be installed.

Place piece B, figure 1, over the bolts in A and in a similar manner place piece C over B. Run the 4 brass nuts down the bolts so that the parts are loosely held together. Pick up the partly assembled chamber by one bolt and insert it between cartilage and outside skin. Make incisions in the cartilage and inside skin so that the 4 bolts protrude. Remove the nuts. Place pieces D and E on the bolts in that order. Replace the 4 brass nuts and screw them down loosely. Suture the incision at each end where it extends beyond the chamber and approximate the skin edges accurately. Complete tightening of the nuts so that piece D will compress and kill the cartilage and skin between C and D. With a sharp knife cut away all tissue inside piece E. Clean the surface of pieces B and C. If the chamber is to be used for transplanting place the additional mica-spacer, not shown in figure 1, on C and cover

it with piece F. Impregnate piece G with hot paraffin and lay it on F. Place the retaining ring, H, on G and hold it in place firmly with the springs and nuts, I. Turn the ear over and cut away the skin over the ring on B so that the cut margin fits the ring closely. Should there be free bleeding place small strips of "Gelfoam" around the ring. When the installation

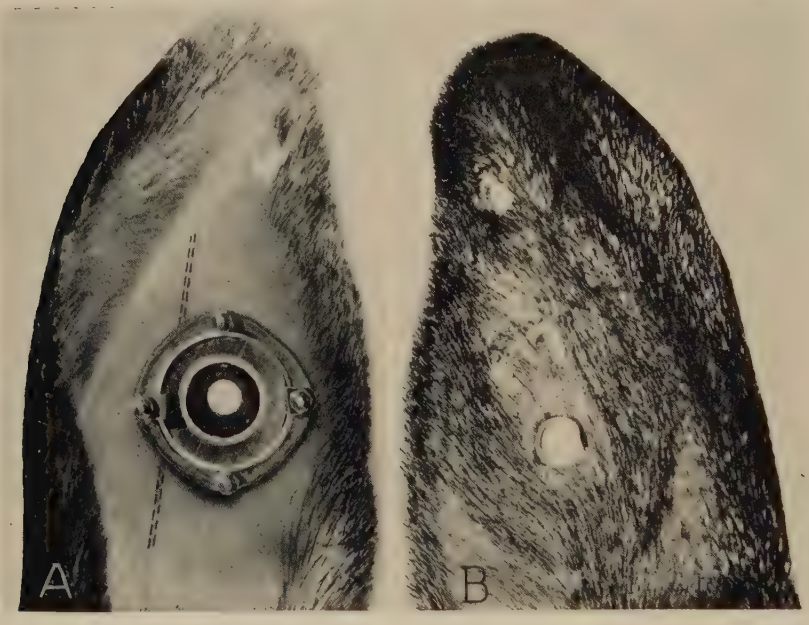

Fig. 2 Photographs, natural size, of an ear with a chamber in it, 5 months after installation. *A* is taken from the inside. The broken lines represent the location of the central artery. The center circle is the observation space filled with vessels which do not show in the photograph. *B* is taken from the outside of the ear.

is complete the projecting gauze portion of the chamber may be moulded through the skin so that it conforms to the shape of the ear. Figure 2 illustrates a chamber 5 months after installation.

Chambers previously in use were most successful only if protected by external splints and covers. These devices are not necessary with the improved chamber. The clamping of

skin and cartilage at the time of installation holds it firmly until vessels and connective tissue grow through the wire gauze from side to side. Without external protection it is possible for animals to scratch the chambers but they rarely do so if there is no motion in any parts of the installations and if no tensions were created by too small an excavation when the chambers were installed.

Once installed chambers require little attention. Originally penicillin was given prophylactically but that was found unnecessary and the practice has been abandoned. Infections occur in about 2% of cases. They generally recover spontaneously providing the animal is left strictly alone.

Successful installations, the ones in which the observation space is completely filled with vessels, are obtained in about 95% of cases. The failures are almost always due to death of the outside skin resulting from defective dissection when the chamber was installed.

As installed chambers age two undesirable things may happen: a portion of the edge may erode through the outside skin or a keloid-like formation may occur around the well on the inside of the ear. For the former there is no remedy. However, no remedy may be needed since such chambers may be useful for as long as two years. For the latter, excision of the growth is all that is necessary. It is of interest that when keloid occurs, its size varies from rabbit to rabbit and also seems to depend upon the operator, being related, at least in part, to the degree of compression of skin and cartilage when the chamber was given its final tightening.

Should it be desirable to subject the tissue in a chamber to fluids of known composition it can be done by using the glass chamber of Abel and Clark ('32) or the tantalum chamber may be readily adapted in the following manner: drill a small hole in the center of a mica cover slip, piece F, figure 1. In an additional mica cover drill two holes, 1 cm apart, and large enough to take an 18 gauge needle. Place two filter paper sealing washers, G, figure 1, together and infiltrate them with Hyrax. Place these on one mica piece and cover them

with the other. The assembled cell is then laid on a hot plate sufficiently warm to melt the Hyrax. When the solvent has been driven off, the mica cell may then be used to replace the cover on chambers filled with vessels. The side with the single hole is placed in contact with the vessels. Fluids may be irrigated through the two holes in the opposite side. These holes when not in use are covered with squares of mica sealed with a mixture of beeswax and rosin. If a small piece of tissue is placed in the hole in the bottom of the cell just before it is applied to the chamber, vessels grow into it and form a tuft within the mica cell. The graft is therefore largely isolated from the vascularized chamber although it receives its own vessels therefrom. The tuft of vessels and cells may be irrigated with solutions of known composition to determine the microscopic effect on the graft and vessels or the fluid may be withdrawn for analysis. The volume of the cell described is .07 cm^3.

To make autogenous transplants into a chamber we have used the following procedure. With the animal anesthetized by intravenous injection of Nembutal and immobilized on its back on an animal board, the ear containing the chamber is extended on the stage of a dissecting microscope. The springs, retaining ring and paper washer are then removed. The cover will invariably stay in place since it is sealed by dried serum at its periphery. The well is filled with 1–500 Metaphen and after 15 minutes the Metaphen is washed away with several changes of Ringer's solution which is finally replaced by Tyrode solution at room temperature. The part from which transplants are to be taken is then exposed and a piece of it, 1 mm^3, excised if the part is not to be removed completely. The wound through which the piece was removed is covered with wet sponges and the piece placed on the stage of a second dissecting microscope in a shallow dish of Tyrode solution at refrigerator temperature about 4°C. The mica cover is then removed under fluid by insinuating the bent end of a needle under the edge and slowly raising it. Using magnification of about 50 × small pieces for grafting are prepared

with steel instruments, forceps and iris scissors, sharpened and pointed as for operations on embryos. It is possible to make grafts of thyroid, for example, so small that only a single follicle will survive. However, there is rarely much point in such a small graft. Grafts from 50 to 200 μ are most satisfactory. Four or 5 of them are laid on the vessels in the chamber, a new mica cover is then placed, and the retaining devices re-applied.

The wound through which the excised part was obtained is then closed. The final procedure consists in adjusting the pressure on the retaining ring in the chamber so that no vessels are occluded. No attempt is made to obtain an immediate return of the original chamber thickness since doing so will result in occlusion of most of the vessels. This return is accomplished later by slow tightening of the nuts, a little each day for several days.

From the time a piece is excised until the final graft is placed on the circulating vessels about 20 minutes or less should elapse. Survival of autografts is the rule and does not depend, in the case of ductless glands, on the presence of a glandular deficiency. Central necrosis of grafts so prepared does not occur. About two weeks are required before the effects of trauma from the grafting procedure subside completely.

The equipment used to study chambers, figure 3, is the same as that devised and assembled by Dr. E. R. Clark and used by him for many years. The animal lies on its back with the head immobilized in a stock (Clark, Sandison and Hou, '31). The ear is extended on the stage of the microscope and rests on a plate of 1/4″ lucite shaped to fit into and be held rigidly by the mechanical stage, the latter being very freely movable. Ordinary stage spring clips fitting in the lucite plate and bearing on the chamber hold the ear in much the same fashion that they would hold an ordinary glass slide. The lucite plate has a center hole in it the size of the condenser opening in the stage. When properly adjusted no respiratory movements are transmitted to the specimen. Should the animal

jump or struggle the free movement of the mechanical stage allows the mounting to follow the ear and no damage to the preparation or the microscope results.

No special optical equipment is necessary. However, with higher magnification it is necessary to select objectives that are as small as possible at the free end in order to be able to examine the entire field without having the objective impinge on the plastic ring of the chamber (piece E, fig. 1).

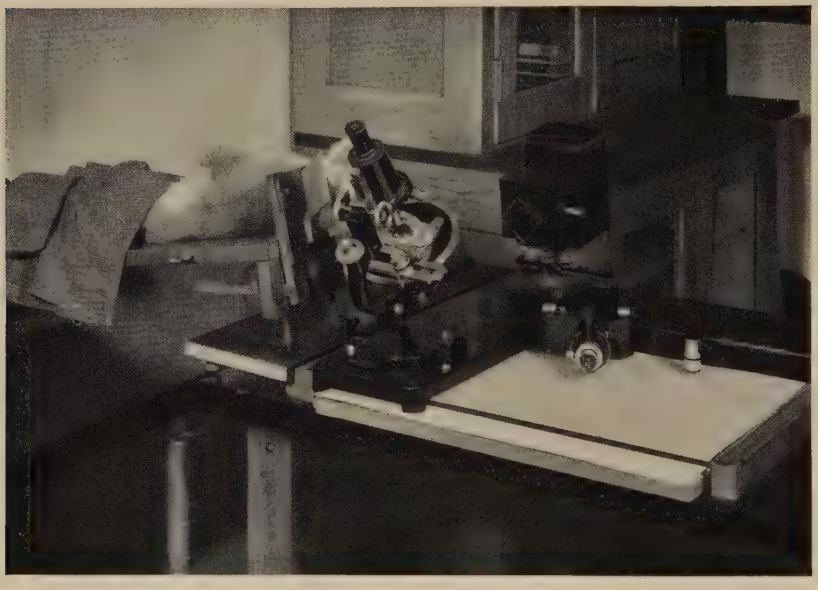

Fig. 3 A photograph of the equipment used for routine study of chambers.

The ordinary substage condenser is used with the top element removed.

The light source for visual and routine photographic work is a 6 v ribbon filament lamp. It is convenient but not necessary to have the light and microscope fixed on a metal base. In any case heat filters must be used. In figure 3, the lamp housing contains a glass cell filled with a very weak solution of copper acetate and in front of this heat resistent glass filters are placed.

For routine photography any portable camera adapted for microscopic use is satisfactory. For cinephotography the apparatus used is that manufactured by Rolab Photo-Science Laboratories, Sandy Hook, Connecticut and adapted for the Cine Kodak Special camera.

The purpose of the transparent chamber method from its inception has been to create in mammals a thin region in which direct microscopic and other types of studies could be made on the same cells for long periods. It is, in effect, a means for duplicating in laboratory mammals as far as microscopic availability is concerned, the naturally occurring thin regions such as are found in frog's webs, tadpole tails, bat wings, etc. Obviously the duplication is not exact since surgery is involved. However, it does provide a very thin layer of living tissue free from epidermis and under conditions that are known. In addition to using the method for studying cells and tissues that grow into chambers, it has been used principally for studying a large variety of transplanted cells some of which are illustrated in figures 4, 5, and 6. In one sense the vessels in chambers provide a naturally renewed culture medium of a composition probably closer to that which the transplanted cells enjoyed normally than can at present be duplicated artificially.

SUMMARY

An improved tantalum and mica chamber is described which when installed in a rabbit's ear permits microscopic studies of living cells and tissues for a period of years. Directions are given for constructing and installing the device and adapting it for exposing the tissues to solutions of known composition.

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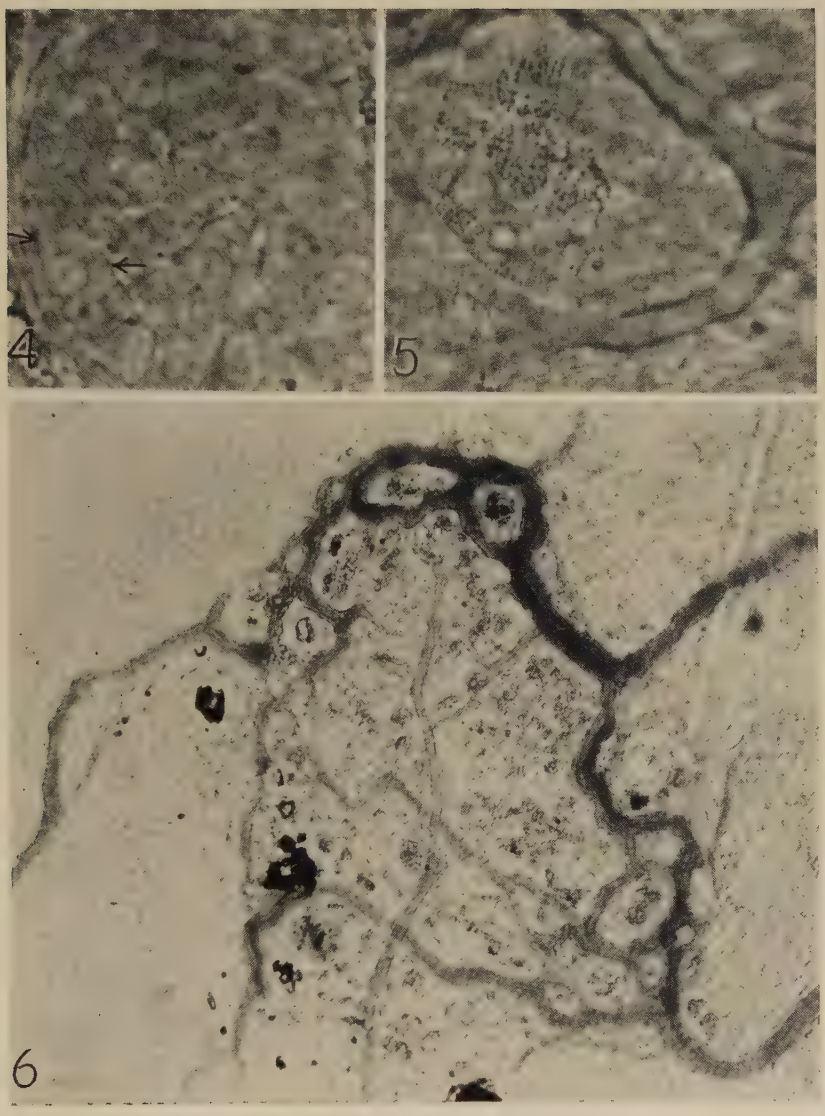

4 and 5 Photomicrographs of living cells in a graft of adrenal cortex, zona glomerulosa. 4 — non-granular cells. 5 — granular cells. × 800.

6 A living graft of lymph node showing the vascular pattern. × 30.

AN ANOMALOUS MUSCLE IN PLACE OF THE STYLOHYOID LIGAMENT OF A CHINESE

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TWO FIGURES

During the dissection of a Chinese man, apparently between 30 and 35 years of age, there was found extending from the styloid process of the temporal bone to the lesser cornu of the hyoid bone, a distinct muscle in place of a stylohyoid ligament on each side of the neck.

DESCRIPTION

In place of the stylohyoid ligaments there are well developed bundles of muscle. Each muscle is a narrow slender band placed between the styloglossus and the external carotid artery, and is superficial to the stylopharyngeus.

The right muscle (fig. 1) arises by a fleshy slip from the tip of the styloid process and passes downwards and forwards, under cover of the hyoglossus. About 1.4 cm from its insertion, it becomes a stout tendon inserted into the lesser cornu of the hyoid bone. The whole muscle measures about 5 cm long and 3 mm wide. It is supplied by a small branch of facial nerve.

The left muscle (fig. 2) takes its origin by a fleshy slip from the posterior surface at the junction of middle and lower thirds of the styloid process. From the tip of the process a very delicate fibrous band accompanies the muscle. These two gradually unite together near their common insertion into the lesser cornu of the hyoid bone. Both have the same course, relations and insertion as those of the right muscle,

but the stout tendon is slightly longer, and the whole muscle is 5.3 cm long and 3 mm wide. It receives its nerve supply from the glossopharyngeal nerve by a small twig.

DISCUSSION

According to the standard textbooks of anatomy the stylohyoid ligament is a fibrous cord which is attached to the tip of the styloid process of the temporal bone and to the lesser cornu of the hyoid bone. It may contain a small piece of cartilage, often may be partially ossified, or may be so slender

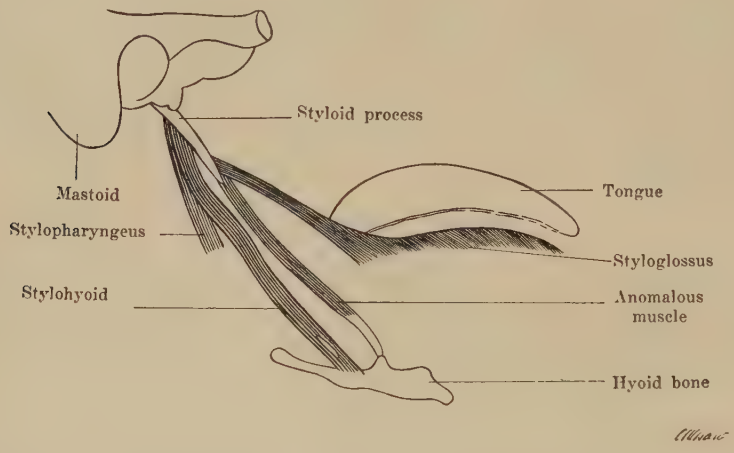

Fig. 1 A semidiagrammatic drawing of the anomalous muscle in place of stylohyoid ligament on the right side, showing the origin and insertion of the muscle, the stylohyoid ligament is not present.

that it escapes notice. Although there are some observers who have reported that some muscular fibers may be found in the stylohyoid ligament, their specimens seem not to be identical with ours because they have not described distinct muscles. Our muscles are definitely not special fasiculi of the stylohyoid muscles which attach to the lesser cornu of the hyoid bone.

From the standpoint of embryology, the stylohyoid is derived from the second branchial arch which connects the

styloid process with the lesser cornu of the hyoid bone; this arch gives rise to the facial muscles and the stylohyoideus. All these muscles are supplied by the facial nerve, so that the right anomalous muscle, supplied by the facial nerve, has undoubtedly come from the same source. The left anomalous is innervated by a branch of the glossopharyngeal nerve, however, so that it seems to be derived from the third arch rather than the second, because the glossopharyngeal nerve supplies the muscle of the third branchial arch.

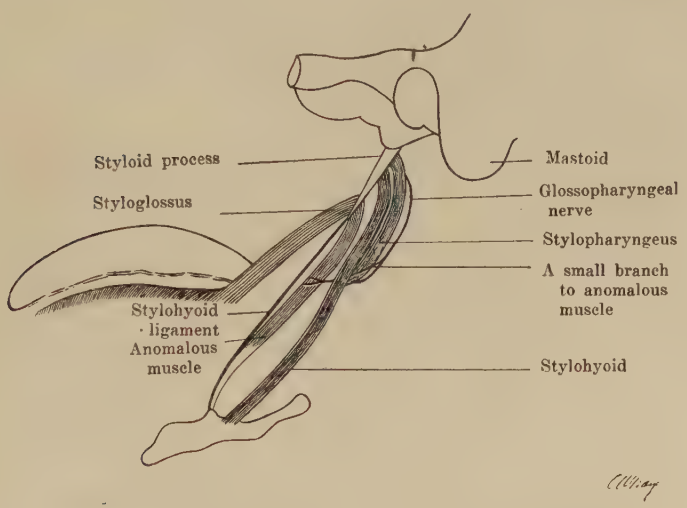

Fig. 2 A semidiagrammatic drawing of the anomalous muscle and a delicate stylohyoid ligament of the left side, showing the relations between the muscle and ligament and nerve supply of the muscle.

The literature available to me fails to give citation for these anomalous muscles in place of the stylohyoid ligaments. During the present hard time, the writer cannot cite all the anatomical literature, but he deems it worthwhile to place the present case on record.

During the dissection of a Chinese man it was found that: The right stylohyoid "ligament" is not present as a fibrous cord, but is a distinct muscle. The left stylohyoid ligament

is still present, but it accompanies and joins with a muscle corresponding to the right one. The two sides have a different nerve supply. The writer would suggest that these two muscles be called accessory stylohyoidei.

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HISTOLOGICAL AND HISTOCHEMICAL STUDIES OF THE NIPPLES OF ESTROGEN TREATED GUINEA PIGS WITH SPECIAL REFERENCE TO KERATOHYALIN GRANULES

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TWELVE FIGURES

INTRODUCTION

The chemical nature of the particulate bodies in the granular layer of the epidermis is poorly understood (Lever, '49). Langerhans first described them in 1882. Waldeyer named them keratohyalin granules because, like "hyalin," they swelled under the influence of weak alkalies, and had a constant relation to the process of cornification (Unna, '13, '28). Macleod's summary in 1903 of the known reactions of these granules has remained essentially unchanged through the most recent edition of his text. More recently, Smith and Parkhurst ('49) have contributed an appreciable volume of data on the tinctorial reactions of keratohyalin.

Our attention was directed to these granules when it was noted that there was a marked increase in the number of such granules in the epidermis of the nipples of stilbestrol-treated guinea pigs.

It is the purpose of this report to bring attention to some of the properties of keratohyalin and to changes in the epidermis and dermis of the nipples of guinea pigs treated continuously with stilbestrol for 14 days.

[1] We wish to express our indebtedness to Dr. E. M. Nadel who initiated this project and actively pursued the studies on estrogen effects on guinea pig nipples.

MATERIALS[2] AND METHODS

Thirty-four stock guinea pigs raised at the National Institutes of Health Breeding Farm were used. Twenty-two of these were virgin females, 6 to 8 weeks old, weighing between 300 to 350 gm. The remainder were males, of similar weight and age.

A stock diet of commercial Purina rabbit chow and fresh cabbage or lettuce was supplemented with carrots once a week.

Pellets of 40% stilbestrol in cholesterol and of 100% cholesterol were prepared and inserted after established methods (Shimkin and White, '41; Nadel et al., '50).

The control animals (12) received cholesterol implants, the experimental animals (22) received stilbestrol implants.

Animals were sacrificed by cranioconcussion and were autopsied. Nipples were immediately removed with a generous portion of the surrounding skin, oriented, and affixed to towelling paper slightly moistened with the fixative to be employed. After a brief interval the towelling and attached tissue were placed in the fixative. Other nipples were affixed to dry towelling and placed in a deep freeze (— 4°C.) box for later use.

Where necessary, control and experimental tissues were processed together by the paraffin method, sectioned (6 μ), mounted and stained on the same slide, or treated simultaneously in the same staining dishes.

Gross differences in the nipples of 10 normal and 12 stilbestrol treated female animals were determined by measuring, for each animal, the area of that longitudinal cross section with the greatest nipple height. For practical purposes, when determining the cross-sectional area, the section of the nipple described was considered to be a triangle. Measurements of the nipples of 22 animals were made to the nearest 0.5 mm (table 1).

[2] We acknowledge our thanks to Dr. Zola Cooper of the Department of Pathology, Washington University School of Medicine, St. Louis, for her help in evaluating the material.

Mitotic counts of the epidermis of 6 animals were based on a total of 5,000 cells for each nipple examined.

Tissues were fixed in absolute ethyl alcohol, alcoholic-formalin, 80% alcohol, Zenker-acetic, sublimate alcohol, and 10% formalin solutions. In addition to the routine hematoxylin and eosin staining method, the following procedures and stains were employed: Microincineration (Cowdry, '48); Verhoeff's elastic tissue stain (Mallory, '38); the Feulgen

TABLE 1

Comparison of nipple area of normal and stilbestrol treated guinea pigs

ANIMAL		WIDTH	NIPPLE HEIGHT	AREA
		mm	*mm*	*mm²*
		Untreated group		
1		1.0	1.0	.50
2		1.0	1.5	.75
3		1.0	2.0	1.00
4		1.5	2.5	1.88
5		1.0	1.0	.50
6		1.5	2.5	1.88
7		2.0	2.5	2.50
8		1.0	1.0	.50
9		1.5	1.5	1.13
10		1.0	1.5	.75
	Mean	1.25	1.70	1.14
		Stilbestrol-treated group		
1		4.5	4.0	9.0
2		4.5	4.5	10.13
3		3.5	5.5	9.63
4		3.0	4.0	6.00
5		3.0	4.0	6.00
6		3.0	3.5	5.25
7		2.5	3.0	3.75
8		2.5	5.0	6.25
9		3.0	3.5	5.25
10		5.0	3.0	7.50
11		3.0	5.0	7.50
12		3.0	5.0	7.50
	Mean	3.38	4.17	6.98

method (Glick, '49); Gormori's alkaline phosphatase method (Ibid.). Periodic acid Schiff reaction (McManus, '48); the gram stain (Glynn, '35); before and after treatment with ribonuclease (Worthington Company); and with toluidine blue before and after treatment with ribonuclease or trypsin (Fisher Company). In addition, a few sections were stained with orcein (Mallory, '38) and by Weigert's resorcin fuchsin method (Cowdry, '48). The leucofuchsin for the Schiff reaction was prepared by the method of Coleman (Glick, '49).

Ribonuclease digestion was carried out using a solution of 0.2 mg of pure crystalline ribonuclease per milliliter of distilled water buffered to pH 7.0. Slides were placed in this solution for three hours at 60°C. (Stowell and Zorzoli, '47) and at pH 5.5 and 37°C. (Lillie et al., '49) after which they were stained with a solution of 0.5% toluidine blue in 20% alcohol. Adjacent sections were placed in a solution similarly prepared by inactivation by boiling.

Trypsin digestion was performed by placing the slides in 0.3% trypsin for three hours at pH 8.3 and at 37°C., with suitable controls as above.

Sublimate alcohol fixed sections were digested in hyaluronidase (Schering) in 0.1 M NaCl at pH 5.3 and at 37°C. for 6 hours.

RESULTS

Gross. Ten normal virgin immature guinea pigs had an average cross-sectional nipple area of 1.14 mm^2. Twelve stilbestrol treated animals had an average cross-sectional nipple area of 6.98 mm^2 (table 1). Nipple widths in the normal group ranged from 1.0 to 2.0 mm with an average of 1.25 mm; height ranged from 1.0 to 2.5 mm with an average of 1.70 mm. In contrast, nipple widths in the stilbestrol treated group ranged from 2.5 to 4.5 mm with an average of 3.38 mm while the nipple heights ranged from 3.0 to 5.5 mm with an average of 4.17 mm.

The nipples of the stilbestrol treated animals were visibly larger and hyperemic, with the base sharply circumscribed,

prominent, and contrasting in color with the surrounding skin. The normal nipples were pale, inconspicuous and hidden by the surrounding body hair.

Microscopic. In contrast with the epidermis of the normal nipples, those of the estrogen treated animals showed acanthosis and hyperkeratosis. The epidermis of the normal nipples varied from 3–8 cells in thickness (fig. 1) while those of the estrogen treated animals varied from 8–16 cells in thickness (fig. 2). Individual cells of the basal and spinous layer of the hormone treated animals were larger than normal. In addition to increase in cell size and number, there was intracellular edema which was more prominent in spinous than in basal cells. Mitotic figures per 5,000 cells were counted in three experimental and three control animals. A total number of 6, 10 and 15 mitoses were counted in the controls, in contrast to 18, 28 and 35 mitoses in the experimental group. Mitotic figures were noted directly above the basal layer which contained only an occasional mitotic figure. Some of the mitotic figures resembled the dark clumped figures associated with colchicine arrest. Binucleated cells were seen not infrequently in the lower third of the spinous layer.

The keratohyalin granules were present in increased numbers in the estrogen treated animals, but there was no apparent alteration in the size, tinctorial qualities or distribution of the granules.

Rete pegs were deeper and broader, and in some areas appeared to have papillary infoldings in the estrogen treated animals. Hair follicles, when present, were plugged with keratin. There appeared to be no difference in the number of hair follicles in the nipples of normal and stilbestrol treated animals.

In the dermis of the stilbestrol treated animals there was increased vascularity. The collagen fibers appeared thicker and more swollen than in the normals and were spread apart by numerous dilated vessels of capillary size. There was a slight perivascular infiltration of mononuclear cells.

Microincineration. In the epidermis of the nipples of the control animals the keratohyalin granules were identified as gleaming white deposits of mineral ash. Between the granular and basal layers there was relatively little white ash; most of the mineral deposits in this zone appeared as blue and blue-gray ash. The basal layer was easily identified by virtue of its high content of white ash (fig. 3). The coarse white ash granules in incinerated preparations have been shown to be essentially calcium ash by Lansing and Scott ('42) while iron is readily detected by its characteristic orange to red color.

In the stilbestrol treated animals the increase in number of keratohyalin granules was not associated with demineralization of the granules. There was considerably less white mineral ash in the spinous layer of the experimental group. The basal cells of normal animals were noticeably richer in white ash content than those of the estrogen treated animals (fig. 4). The iron content of normal skin was very low and there was no apparent deviation from this in the hyperplastic skins.

Verhoeff's elastic tissue stain. With Verhoeff's method, the keratohyalin and the elastic fibers were colored a dense black (fig. 5) which persisted after vigorous differentiation in 2% ferric chloride solution had completely decolorized all nuclei. Keratohyalin granules were more prominent in the experimental group (fig. 6). This appeared related to an increase in the number of cells and granules in the granular layer and not to an increase in staining intensity per granule. Elastic fibers were heavier and more prominent in the deep elastic layer of the dermis in estrogen treated animals (fig. 7, 8). Individual fibers appeared thicker but not longer than in the controls. Many fibers had knobbed ends. There appeared to be no qualitative alteration in the subepidermal elastic plexus. Because of the total increase in the size of the nipple, more elastic fibers appeared to be present in the experimental group. A quantitative increase in elastic fibers per unit area could not be demonstrated.

Feulgen reaction. In all the sections examined, the keratohyalin granules did not colorize after exposure to the Feulgen reagents. The nuclei of the various epidermal cells in control and experimental animals showed no significant variation in the intensity of the Feulgen reaction.

Periodic acid Schiff technique (PAS). The basement membrane and stratum germinativum were colored an intense cherry red after treatment by the PAS method. Cells of the basal, spinous and cornified layers in the control and experimental animals showed little or no PAS reaction. The keratohyalin granules were variable in their reaction; only one-third of the specimens of both groups reacted positively. The variation in this reaction was not dependent on the method of fixation. Elastic fibers were colored deep red after treatment with leucofuchsin alone.

Toluidine blue and enzymatic digestion. The keratohyalin granules and elastic fibers were metachromatic, when stained with toluidine blue, in the control and experimental groups. There was diminished staining intensity in those tissues fixed in absolute alcohol, but metachromasia was still evident. No loss of toluidine blue uptake or of metachromasia after ribonuclease or hyaluronidase incubation was observed. Complete dissolution of the keratohyalin granules occurred in both control and experimental groups after the nipple sections were incubated with trypsin.

Gram stain. With the gram procedure the keratohyalin granules and the cornified layer both stained a deep violet which resisted decolorization with acetone. In sections previously incubated with ribonuclease, there was no alteration in the gram reaction of the granules, but the cellular elements of the dermis were decolorized.

Alkaline phosphatase. In both the control and experimental groups there was definite blackening of the granular layer. This was confined specifically to the granules, both free and those associated with nuclei. The reaction was weak in the controls (fig. 9), and markedly positive in the stilbesterol treated animals (fig. 10).

The dermal papillae were markedly positive in the estrogen treated animals in contrast with the negative reaction of the controls. The intensity of the reaction however was less than that of the mammary glands which were densely black in both control and experimental group. Blackening could not be demonstrated in association with a particular cell type in the connective tissue, but appeared as deposits between collagen fibers. The reaction was not sharply confined to the dermis for there was a greyish cast to the basal layer as well (fig. 10). Hair follicles and sheaths stained with increased though variable intensity. The sebaceous glands stained strongly as did the endothelial cells of the capillaries.

DISCUSSION AND CONCLUSIONS

Nipple growth in the guinea pig has been carefully studied by many investigators. Enlargement of this specialized portion of the skin has been described after estrogenic and androgenic stimulation. Such growth is reported to be independent of the pituitary gland (Folley and Malpress, '48). Gross (Jadassohn et al., '38) and microscopic (Uehlinger et al., '41) changes of the epidermis of the estrogen treated nipple have been described. These changes include hyperkeratosis, acanthosis and an increase in epidermal width to 4–5 times that of control animals after 5 days of local estrogen treatment, and to 6–8 times after 30 days of such treatment. Our observations are in accord with these.

In addition, in 6 random samplings of mitotic activity, we have noted a 2.7 fold increase in the number of mitoses in the nipple epidermis of the estrogen treated animals, while others (Jadassohn et al., '38) have reported that "scarcely a mitosis is to be seen" in either normal or estrogen treated animals unless colchicine were given in addition to the hormone. Although only a 2.7 fold increase in mitotic figures could be detected, a 6-fold increase in total nipple area was observed in the estrogen treated animals. We attribute this difference to increased cell size, and to the extra- and intracellular edema observed in the hormone treated animals.

An explanation for the reaction of the nipple epidermis is not immediately available, but may be related to the similar origin of the nipple area and the so-called sex skin as described by Zuckerman ('40).

It would appear that histological evidence of increases in alkaline phosphatase, in elastic fibers and in vascularity are associated with the increased nipple growth observed in the stilbestrol treated animals. Folley ('40) has reported that the guinea pig mammary gland is particularly rich in phosphomonesterase A. Sections stained for alkaline phosphatase demonstrated marked blackening in the mammary glands of both control and experimental animals.

Studies on alkaline phosphatase localization in the guinea pig nipple with increases after estrogen treatment have not been reported to our knowledge. Others, however, have reported localization of this enzyme in the granular layer of normal guinea pig epidermis (Bourne, '44) and in normal and pathological human epidermis (Fisher and Glick, '47). That the keratohyalin granules were associated with calcium and/or magnesium ash and with alkaline phosphatase activity and were more abundant in the estrogen treated animals may be significant. In other species and tissues, estrogen activity is intimately associated with calcium metabolism, as well as with alkaline phosphatase activity (Lurie et al., '49; Moog, '46).

The apparent increase in elastic fibers in the nipple dermis of the experimental group is in accord with the reports of others. Emerson et al. ('48) has described an increase in the elastic tissue in sections of the breasts of women treated with stilbestrol for mammary carcinoma. Goldzieher ('46) has reported increases in the elastic tissue in sections of the skin of senile people who had been treated with estrogens. However, Gross ('49) by electron microscopic studies has indicated that, contrary to former belief, the elastic fiber is a two component system and is not homogeneous. The bundles are composed of characteristic trypsin-resistant threads and a trypsin-sensitive heat-resistant "amorphous" binding ma-

trix. Inasmuch as estrogens are known to enhance the accumulation of ground substances (Chain and Duthie, '40) and extracellular fluid (Taylor and Sprunt, '43) in connective tissues related to elastic fibers, there is the possibility that these hormones may also act upon the components of the elastic fibers, and thus appear to effect an increase in stainable elastic elements.

Normal epidermis is known to have a high content of calcium (Lansing et al., '48b) which by means of radiocalcium studies (Lansing et al., '48c) is shown to be actively exchanged. Our microincineration studies indicate an apparent decrease in the amount of calcium and/or magnesium ash contained in the basal and spinous cells of the hyperplastic nipples. This is consistent with the general observation that young actively growing cells have a low mineral content while mature or old cells have a high mineral content (Lansing, '42). It is interesting to note that in experimental carcinogenesis there is likewise a reduction in epidermal calcium content (Lansing et al., '48b). An increase in the content of alkaline phosphatase in the basal layer (Biesele and Biesele, '44) and in underlying connective tissues has likewise been reported in healing after methylcholanthrene injury (Ibid.) or after cuts and burns (Fell and Danielli, '43).

In addition to their association with calcium and/or magnesium and alkaline phosphatase, keratohyalin granules are Feulgen negative and therefore presumably do not contain desoxyribonucleic acid (Stowell, '46). That the granules are gram positive has been previously mentioned (Lillie, '48). Gram positivity, according to Dubos ('45) is determined by superficial ribonucleoprotein substances. However, the granules were not digested by ribonuclease. This would appear to indicate the absence of unaltered low polymers of ribonucleic acid (Michaelis, '47) in the granules. The metachromatic reaction with toluidine blue would indicate, according to Michaelis (Ibid.) and Sylvén ('41) the probable presence of sulfuric esters of high polymeric carbohydrates, high poly-

meric carbohydrates with carboxyl groups, or polymeric metaphosphate.

Our observations on the tinctorial reactions of keratohyalin granules differ in several respects from those of Smith and Parkhurst ('49):

1. Using microincineration, we determined that the granules are essentially free of iron and are rich in calcium and/or magnesium. Smith and Parkhurst ('49) implicate iron and doubt the presence of calcium. The latter observation, however, was based upon the almost completely unreliable reaction of alizarin red S for calcium.

2. We have found keratohyalin to be essentially resistant to ribonuclease whereas Smith and Parkhurst ('49) find marked digestion of the granules by this enzyme. It is possible that this difference in our observations may be due to the use of enzymes varying in protease content.

Except for these differences in observations, our findings to a large extent confirm those of Smith and Parkhurst, '49).

In many respects, keratohyalin and elastic fibers appear to have similar properties. Both have a pronounced affinity for calcium (Lansing et al., '48a). Both contain, or yield on hydrolysis, an aldehyde fraction, and stain intensely with the Verhoeff technique. In addition both are digested in part by trypsin and resist hydrolysis by weak bases. The differences in their degree of affinity for resorcin fuchsin and orcein and their dissimilar staining reaction in the hematoxylin and eosin method may be related to the presence of two distinct but intimately associated components of the elastic fibers.

SUMMARY

1. The nipples of 34 guinea pigs that were treated with stilbestrol for 14 days were examined both histochemically and histologically. Special attention was given to the keratohyalin granules.

2. After 14 days of stilbestrol treatment, the guinea pig nipple increased 6-fold in total area, but less than 3-fold increase in mitotic figures was noted. This disparity may

be attributed to increased cell size and intra- and extracellular edema.

3. Associated with estrogen hyperplasia, there is hyperkeratosis, acanthosis, and an increase of mitotic figures. Microincineration studies indicated that this is associated with a decrease in the content of calcium ash in the basal layer of the epidermis.

4. The relationship of estrogens to the alkaline phosphatase, and calcium alterations is discussed.

5. Keratohyalin granules increase in number with estrogen treatment and are associated with calcium and/or magnesium deposits (as seen by microincineration) and with alkaline phosphatase. In addition the granules are Feulgen negative, gram positive, periodic acid Schiff positive, metachromatic with toluidine blue and stain intensely with the Verhoeff technique. They resist hydrolysis with weak bases, are not digested by ribonuclease or hyaluronidase but are digested by trypsin. These reactions are similar to many of the reactions of elastic fibers.

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PLATE 1

EXPLANATION OF FIGURES

1 Normal guinea pig nipple. Hematoxylin and eosin stain. × 80.

2 Estrogen stimulated guinea pig nipple. Hematoxylin and eosin stain. × 80.

3 Microincineration of normal guinea pig nipple. Dark field illumination. × 250.

4 Microincineration of estrogen stimulated guinea pig nipple. Dark field illumination. × 250.

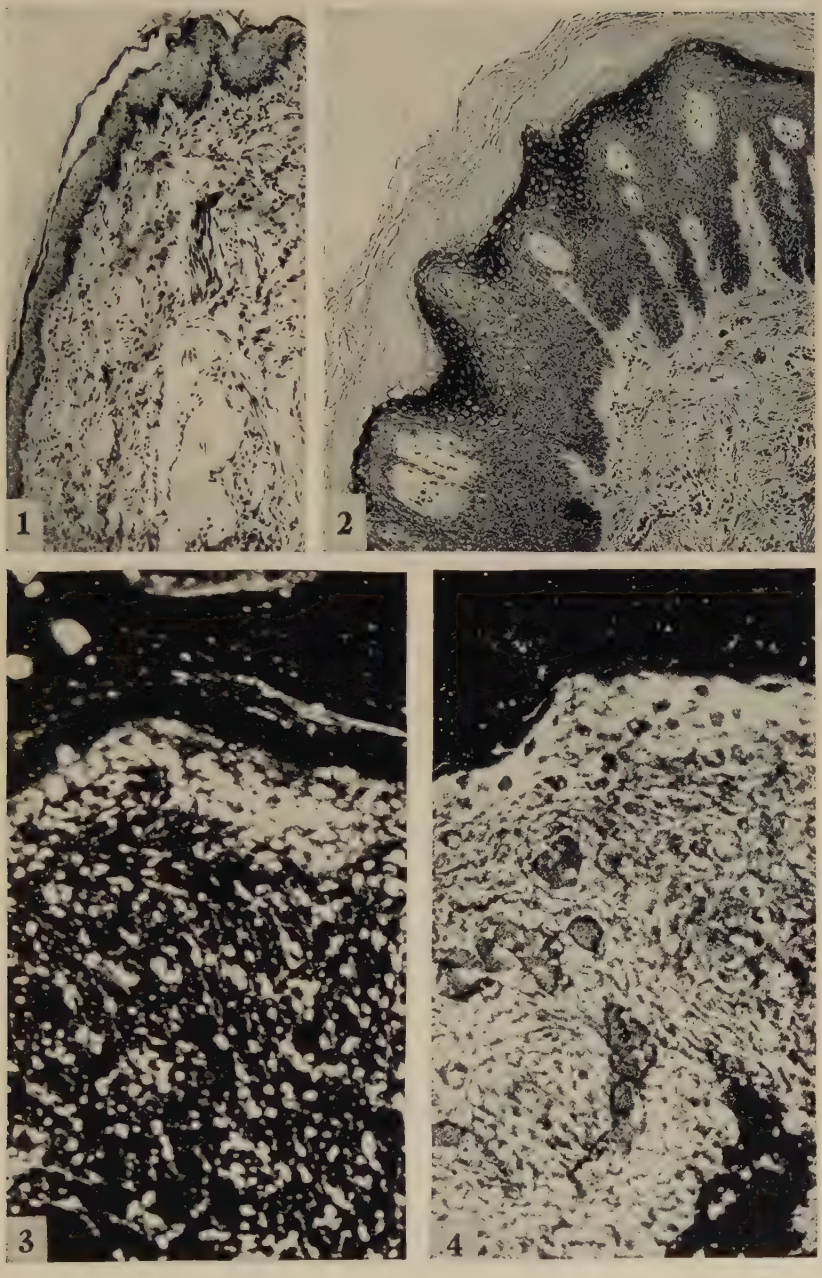
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PLATE 2

EXPLANATION OF FIGURES

5 Normal guinea pig nipple. Surface area. Verhoeff elastic tissue stain. × 250.

6 Estrogen stimulated guinea pig nipple. Surface area. Verhoeff elastic tissue stain. × 250.

7 Normal guinea pig nipple. Deep area. Verhoeff elastic tissue stain. × 250.

8 Estrogen stimulated guinea pig nipple. Deep area. Verhoeff elastic tissue stain. × 250.

5

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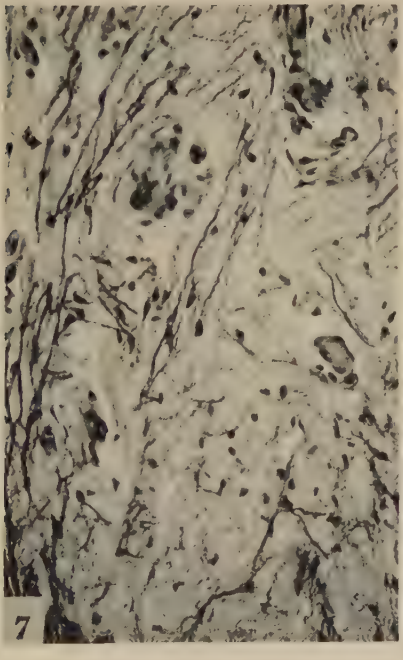
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PLATE 3

EXPLANATION OF FIGURES

9 Normal guinea pig nipple. Gomori alkaline phosphatase procedure. × 60.

10 Estrogen stimulated guinea pig nipple. Gomori alkaline phosphatase procedure. × 60.

11 Normal guinea pig nipple. Gomori alkaline phosphatase procedure. × 280.

12 Estrogen stimulated guinea pig nipple. Gomori alkaline phosphatase procedure. × 280.

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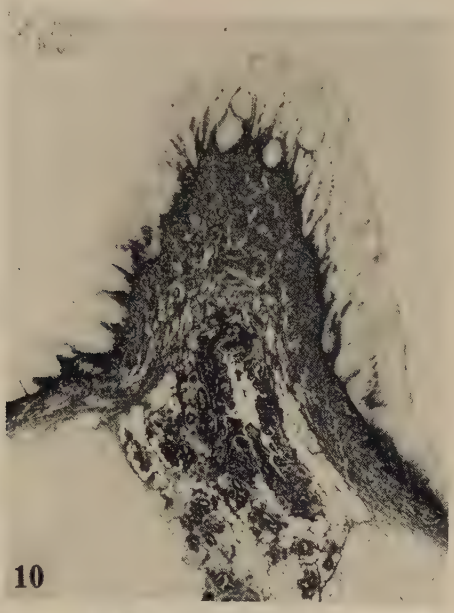
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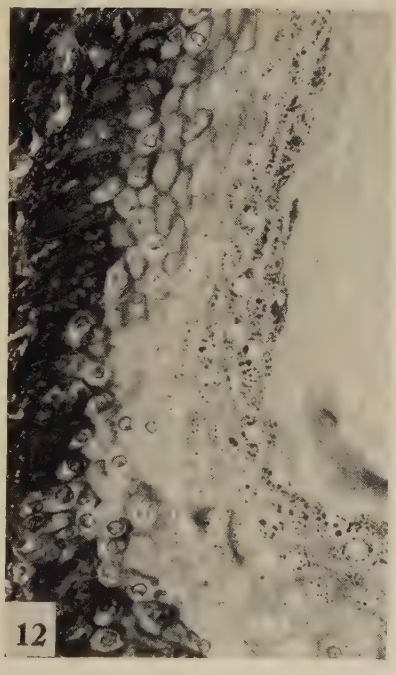
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DIFFERENCES IN THE RESPONSE TO GROWTH HORMONE OF THE RAT'S PROXIMAL AND DISTAL TIBIAL EPIPHYSES [1]

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It has been shown that when the administration of pituitary growth hormone to normal rats was started at an age when closure of the epiphysis of the third metacarpal was imminent (81 days), this bone not only failed to grow but underwent epiphyseal closure at the normal time (90–110 days) during the injections of the hormone (Asling, Becks, Simpson, Li, and Evans, '48). However, as reported, these rats increased in weight and total body length as expected.

It was important to determine whether the metacarpal was unique or whether some other epiphysis might be similarly unresponsive under the same circumstances. The tibia is well adapted to such a study. It has always been possible by injection of growth hormone to demonstrate maintenance of growth at its proximal epiphysis after hypophysectomy, and even to reactivate it after growth had ceased, in the normal adult as well as in the hypophysectomized dwarfed rat (Evans, Becks, Asling, Simpson, and Li, '48; Becks, Simpson, Evans, Ray, Li and Asling, '46). The distal tibial epiphysis, however, is reported to undergo epiphyseal closure at approximately the same time as does the metacarpal epiphysis (Dawson, '25). In the histologic study of tibial growth it was important to determine whether both epiphyses would contribute to the elongation of the bone or whether the distal epiphysis, like that of the metacarpal, would be unresponsive and even

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undergo epiphyseal closure while the proximal epiphysis was responding to the growth hormone's stimulus. In addition to this main objective it was also proposed to study cross sections of the shaft of the tibia in order to detect growth in diameter.

The experimental conditions were described in the earlier report (Asling et al., '48). In brief, they were as follows: three groups of normal female rats of the Long-Evans strain (6 rats per group) received pure pituitary growth hormone starting at the age of 81 days. The dose was 1 mg per day. In an attempt to increase its effectiveness it was divided and injected twice daily. These injected rats were sacrificed at three successive 10-day intervals, so that they were 91, 101, and 111 days of age, respectively, at autopsy. Four groups of uninjected controls were used; one was sacrificed at the onset of the experiment while the other three groups were sacrificed at the same intervals as the injected rats. As has already been reported, the increase in body weight and length of the injected rats was substantially accelerated even in the group sacrificed at the first 10-day interval. This accelerated growth was maintained throughout the 30-day injection period.

The freshly dissected tibias were measured, fixed in neutral formol, and decalcified. Histologic sections of the proximal end of the bone were cut in the median sagittal plane. The distal end was cut in the frontal plane in order to demonstrate the epiphyses of both tibia and fibula.[2] Transverse sections were cut through the center of the shaft.

The results of the experiment are shown in a series of tables.

Table 1 shows the average length of the tibia in the various groups. The tibia lengths of injected rats exceeded those of controls in every age group; in the second and third group (after 20 and 30 days of injections) the differences attained were statistically significant.

It should be noted that the length reached after 30 days of injection was equal to the normal adult length of the rat

[2] In the rat, the tibia and fibula are fused along their distal one-third; the proximal two-thirds are separated.

tibia.[3] Maintenance of this accelerated growth would have resulted in gigantism. This can occur, as shown by Evans et al. ('48).

In the histologic examination of the proximal tibial epiphysis, evidences of increased reactivity were sought not only by examination of the cartilage plate and the subjacent bony trabeculae, but also by measuring the width of the plate. The average widths for the various groups are shown in table 2.

As expected, the plates were wider in the growth hormone-injected rats. The maximum reactivity was reached within

TABLE 1

Average length of tibia of normal female rats injected with growth hormone for 10, 20, and 30 days, starting at 81 days of age

AGE AT AUTOPSY	LENGTH OF TIBIA [1]	
	Growth hormone treated [2]	Controls
days	*mm*	*mm*
81		35.5 ± 0.55
91	37.6 ± 0.29	36.7 ± 0.35
101	38.7 ± 0.21	36.8 ± 0.31
111	39.8 ± 0.22	37.6 ± 0.51

[1] Averages and standard errors, 6 rats per group.

[2] Electrophoretically homogeneous pituitary growth hormone, 1.0 mg daily, divided into two doses.

10 days and did not increase thereafter; nor was this reactivity reduced with prolongation of the injection period to 30 days.[4]

By study of the data for uninjected rats the gradual narrowing of the cartilage plate with increasing age may be

[3] The length ultimately attained in normal rats is 39.6 ± 0.51 mm at 250 days of age; it is no longer (39.6 ± 0.76 mm) at 650 days of age (Becks, Asling, Simpson, Li and Evans, '49).

[4] The plate was somewhat narrower in the 20-day injection group than in the 10-day and 30 day group; the controls for this group were correspondingly reduced. This may reflect the fact that several of these 101-day-old rats had minor respiratory infections during the two days preceding autopsy; their growth may have been retarded during this period.

seen. Although the amount of reduction during the ages spanned by this experiment (81–111 days) was not enough to be significant by statistical tests, it nevertheless corresponded to that anticipated on the basis of the survey report of Becks, Simpson and Evans ('45).

The only important histological differences in the proximal epiphyseal plates of the injected rats from those of their controls were an increased length of the columns of proliferating chondrocytes and a slightly wider zone of vacuolated cells, both indications of cartilage activation. Otherwise, as respects the finding of multiple marrow tufts at the line of erosion

TABLE 2

Width of proximal epiphyseal cartilage plate of tibia of normal female rats injected with growth hormone for 10, 20, and 30 days, starting at 81 days of age

AGE AT AUTOPSY	WIDTH OF EPIPHYSEAL PLATE	
	Growth hormone treated	Controls
days	*micra*	*micra*
81	..	184 ± 14.7
91	227 ± 17.4	162 ± 10.2
101	184 ± 5.0	169 ± 5.2
111	210 ± 12.8	147 ± 14.0

of cartilage, the presence of epithelioid rows of osteoblasts, and the formation of numerous delicate bony trabeculae in the primary spongiosa, no marked differences could be demonstrated.

In the examination of the distal end of the bone, the epiphyseal plates of both tibia and fibula could be seen. They were examined for evidences of growth activity and for maturation (epiphyseal closure). Separate records were made for tibia and fibula, the former having been reported by Dawson as closing slightly earlier. Table 3 shows the epiphyseal status in the various groups; intact cartilage plates are recorded as +, perforated plates as ±, and complete epiphyseo-dia-

physeal fusion as —. A separate entry appears for each animal, as these attributes cannot be treated statistically.

As shown by the table, athough the majority of animals had intact cartilage plates at the onset of injections, epiphyseal fusion took place during the injection of growth hormone.[5] This was consistent with the earlier observations on the third metacarpal of these same rats. Even when intact plates could be found in the injected rats they showed no evidences of activation.

TABLE 3

Status of the distal epiphyseal cartilage plates of the tibia and fibula of normal female rats injected with growth hormone for 10, 20, and 30 days starting at 81 days of age

AGE AT AUTOPSY	STATUS OF DISTAL EPIPHYSEAL PLATE [1]			
	Tibia		Fibula	
	Growth hormone treated	Control	Growth hormone treated	Control
81	. . .	+ + + + ± ±	....	+ + + + ±
91	+ — — — — —	± ± ± ± — —	± ± ± — — —	± ± ± ± ± ±
101	— — — — — —	± — — — — —	± — — — — —	± ± — — —
111	— — — — — —	— — — — — —	+ — — — — —	— — — — — —

[1] Each symbol represents the epiphyseal plate of an individual animal, as follows: + intact, ± perforated, — absent.

The status of the epiphyseal plates in the uninjected rats is of interest since it shows that the time of closure of these epiphyses in the Long-Evans strain of rat closely approximates the age of closure reported for the Wistar albino strain. Dawson ('25) reported distal tibial closure to start at 92 days and be complete at 98 days of age. He found the fibula to close slightly later, starting at 98 and completing at 130 days

[5] Not only was closure not delayed by the injection, but comparison with the controls of the same age suggested a slight acceleration of epiphyseal fusion in the injected rats. However, since the metacarpals had shown just the reverse (i.e., slightly greater proportions of intact plates in the injected rats) the differences are probably not significant, and it is most likely that growth hormone had no effect on epiphyseal closure in this experiment.

TABLE 4

Area of marrow cavity and cortical bone in cross sections of the tibial shaft of normal female rats injected with growth hormone for 10, 20, and 30 days, starting at 81 days of age

AGE AT AUTOPSY	ACTUAL AREA				PERCENTAGE OF TOTAL AREA			
	Growth hormone treated		Controls		Growth hormone treated		Controls	
	Marrow	Bone	Marrow	Bone	Marrow	Bone	Marrow	Bone
days	mm^2	mm^2	mm^2	mm^2	%	%	%	%
81	..	..	1.0 ± 0.10	2.0 ± 0.15	...	...	34.0	66.0
91	1.7 ± 0.17	2.5 ± 0.14	1.3 ± 0.06	2.3 ± 0.09	41.6	58.4	35.8	64.2
101	1.2 ± 0.06	2.6 ± 0.08	1.6 ± 0.16	2.3 ± 0.12	31.9	68.1	39.9	60.1
111	2.0 ± 0.22	2.9 ± 0.14	1.4 ± 0.09	2.4 ± 0.16	41.3	58.7	38.1	61.9

of age. The histologic studies reported here indicated that tibial epiphyseal closure was complete in half of the group at 91 days or shortly thereafter, while fibular closure followed by a very few days, complete in half the group by or just before 101 days of age.

Cross sections of the tibia through the center of its shaft were studied to establish whether the increased periosteal osteogenesis seen with growth hormone would be accompanied by endosteal bone resorption and marrow cavity expansion, or whether the cortical bone would increase in thickness through lack of internal resorption. Histological evidences of apposition and resorption were sought. In addition, to obtain a quantitative expression of these phenomena, tracings were made of the sections at 12.5-fold magnification and measured with a planimeter. Two areas were thus obtained — that of the entire bone (whose perimeter was the periosteum) and that of the medullary cavity (whose perimeter was the endosteum). A third area, that of the section of cortical bone, was calculated by subtracting the area of the medullary cavity from the total area.

The diameter of the shaft of the tibia increased markedly through periosteal osteogenesis in the injected animals. Expansion of the marrow cavity was equally rapid, and as shown in table 4, the ratio between the area of marrow cavity and of cortical bone was virtually a constant. The cortical bone, constituting 66% of the total area of the section at the onset, remained between 58.5% and 68.1% in all groups, injected as well as control, during the experimental period. Thus the cortical bone did not undergo appreciable thickening, since it was removed endosteally in pace with its periosteal formation. The absolute areas of marrow and of bone, however, were greater in the injected animals. The increase was consistently greater in the longer injection periods, though not statistically significant. The rate of activity, both in periosteal apposition and endosteal resorption of bone, was slow, so that no outstanding differences in osteoblastic and osteoclastic activity could be demonstrated between injected and control groups.

DICUSSION

It appears from this study that the two epiphyses of the tibia must follow different laws in their growth, since they differed so markedly in their response to growth hormone. A potential for continued elongation of the tibia existed at the beginning of the experimental period, as demonstrated both by the fact that at this time the bone was far short of its definitive (maximum normal) length, and by the observed growth in the uninjected controls during the experimental period. Although both epiphyses had cartilage plates at the onset, only one of these — the proximal — was activated by the growth hormone injections. The distal epiphyseal plate not only was unresponsive, but epiphyseal closure took place during the injections. Apparently after 81 days of age, or perhaps earlier, the entire potential for growth of the bone was vested in the proximal epiphyseal cartilage plate. There are characteristic differences in the behavior of these plates in normal rats, the distal epiphysis undergoing fusion between 90 and 100 days of age, while the proximal plate normally ceases its growth activity somewhere near 250 days of age, and the bone does not normally grow further although the plate persists far beyond that age. It may, however, be activated to overgrowth by administration of growth hormone. When growth hormone was administered to hypophysectomized rats for 14 months, the tibias exceeded normal length by 10% (Evans et al., '49). The proximal epiphysis was still reactive at the end of the experiment. However, as in the present experiment, the distal epiphysis had closed during the injections. No satisfactory explanation for the differing behavior of these two epiphyses is available at present.

SUMMARY

The injection of growth hormone in 81-day-old normal female rats of the Long-Evans strain resulted in marked growth of the tibia. All of the growth could be attributed to activation of the proximal epiphyseal cartilage plate, which increased in width significantly during the first 10 days of in-

jections and still maintained this greater width after 30 days of injections. The distal epiphysis, on the other hand, underwent complete epiphyseal fusion during the injections. This fusion occurred at the normal time, approximately 91 days of age. The distal fibular epiphysis closed slightly later, but before 101 days of age, as was also normal.

Examination of cross sections of the shaft showed that periosteal bone apposition had resulted in increased diameter of the bone, but that endosteal resorption had kept pace with this increase.

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A STUDY OF THE PRECIPITATING EFFECTS OF BASIC FIXING SOLUTIONS [1]

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Both fixatives and biological tissues have complex chemical structures which make the scientific approach difficult. Today, there are 20 or more basic fixing solutions utilized in making many combinations. We felt that some knowledge of the effect of pH and other factors on coagulation might be gained by studying the action of the commonly used ones on emulsions made from liver and brain. These two organs were selected both because they have different chemical compositions and because they are available in relatively large amounts. The problem has been approached from the standpoint of the quantity, the character and the specific gravity of the precipitates.

In each experiment, 40 gm of tissue were thoroughly emulsified in 200 cm^3 of distilled water with an Osterizer Mixblender. This was then filtered with coarse-meshed cheese cloth. Two cubic centimeters of the filtrate (0.4 gm tissue) were added to 13 cm^3 each of the following fixing reagents and buffered pH solutions.

A. Primary fixing solutions — acetic acid (5%); nitric acid (1%); chromic acid (1%); picric acid (sat. aq.); mercuric chloride (6.9%); concentrated formalin; potassium dichromate (3%); osmic acid (1%); platinic chloride (1%) and finally absolute ethyl alcohol.

[1] Aided by a grant from the Committee on Scientific Investigation of the American Medical Association.

B. Buffered pH solutions — the pH of each of the 10 fixing fluids above was determined and buffered solutions of identical hydrogen-ion concentrations were made up according to the formulae of Johnson and Lindsey ('39). This was done to determine the effect of the pH alone on protein precipitation.

TABLE 1

Shows the average amount of precipitate in cubic centimeters occurring in 10 primary fixing solutions and in fluids buffered to the same hydrogen-ion concentration. In each case, 2 cm³ of emulsified tissue (liver or brain, 0.4 gm) were put in 13 cm³ of test solutions

FIXING SOLUTION	pH	LIVER			C. N. S.		
		Ppt. fixing sol.	Ppt. buffer sol.	Diff.	Ppt. fixing sol.	Ppt. buffer sol.	Diff.
Nitric A.	0.88	0.13	0.1	0.03	1.32	1.05	0.27
Chromic A.	1.12	0.48	0.23	0.25	0.81	1.08	— 0.27
Picric A.	1.33	0.73	0.20	0.53	0.81	1.13	— 0.32
Plat. Chlor.	1.48	0.86	0.11	0.75	0.78	1.23	— 0.45
Acetic A.	2.32	0.16	0.25	— 0.09	1.52	0.99	0.53
Merc. Chlor.	2.95	0.76	0.73	0.03	0.58	0.72	— 0.14
Formol	3.15	1.27	0.70	0.57	1.57	0.63	0.94
Pot. Bichrom.	3.92	1.11	0.66	0.45	1.33	0.62	0.71
Osmic A.	6.2	0.20	0.33	— 0.13	0.68	0.76	— 0.08
Alcohol	8.2	0.73	0.10	0.63	0.35	0.33	0.02

The final step was to centrifuge the emulsions in tubes for 10 minutes with an International Centrifuge regulated to a speed of 2,200 revolutions per minute. The measurements of the quantity of precipitates were taken from the graduated test tubes. By the above procedures, it is possible to check, at the same time, both the precipitating effect of various fixing fluids and that of the controlled buffered solutions.

The tissues of 5 dogs, three monkeys and three cats were utilized in the experiments.

RESULTS

Significant quantitative variations are noted between the precipitates from liver and brain emulsions both in fixing and buffered solutions (table 1). With only 0.4 gm of tissue used in each 13 cm^3 of the fixing fluid, the amount precipitated varies from 0.13 to 1.27 cm^3 for the liver emulsion and from 0.35 to 1.57 for that of the brain. The average value for the liver series is 0.69 and for the brain 1.01. Such solutions as nitric and acetic acids have very little effect on liver whereas the opposite is true on the brain. Osmic acid gives near minimal results in each. Absolute alcohol has a relatively low value on tissues of the central nervous system. Both concentrated formalin and potassium bichromate give comparatively high figures on both. Picric acid, mercuric chloride and platinic chloride are intermediate between the lowest and highest.

In the buffered solutions, the figures are higher for the brain, the range being from 0.33 cm^3 to 1.23 with a mean of 0.86 whereas in the liver the corresponding values are 0.1 to 0.73 with an average of 0.34 cm^3. In 7 out of 20 instances, the buffered fluids precipitate a greater amount than do the fixing solutions (table 1). This is especially true in the brain series. In the liver, a larger amount of precipitate is obtained within the pH range 3.0 to 4.0 whereas in the central nervous system maximal values occur in the more acid buffers between 0.9 and 1.5.

In regard to the character of the precipitates, nitric, acetic and osmic acids give coagulative effects similar to those in corresponding buffer solutions. The granules seem to be fine, dispersed, and of low specific gravity. Picric acid, platinic chloride, formol, potassium bichromate and absolute ethyl alcohol are intermediate in type and produce slightly coarser-grained masses. The precipitates having the highest specific gravities occur in chromic acid and mercuric chloride solutions.

The coagulative power of the basic fixing fluids seems to be reflected when they are mixed to make well-known combina-

tions. Any fixatives, for example, which use concentrations of formol or potassium bichromate show higher volumes of precipitate. Such compounds as Regaud's, Altmann's and Formol-Müller's fall into this category (table 2). On the other hand, Gilson's and Flemming's which possess neither potassium bichromate or formol have relatively low precipitating powers as far as volume is concerned.

TABLE 2

Shows the average amount of precipitate in cubic centimeters occurring in 10 common fixatives and in fluids buffered to the same hydrogen-ion concentration. In each case 2 cm³ of emulsified tissue (liver or brain, 0.4 gm) were put in 13 cm³ of test solutions

FIXING SOLUTION	pH	LIVER			C. N. S.		
		Ppt. fixing sol.	Ppt. buffer sol.	Diff.	Ppt. fixing sol.	Ppt. buffer sol.	Diff.
Gilson	1.0	0.73	0.15	0.58	1.2	1.11	.09
Flemming	1.1	0.87	0.12	0.75	.97	1.04	— .07
Bouin	1.5	0.95	0.19	0.76	.78	1.15	— .37
Champy	1.7	1.03	0.19	0.84	.95	1.14	— .19
Zenker	2.3	0.51	0.37	0.14	.43	.98	— .55
Helly	3.6	0.98	0.70	0.28	.61	.67	— .06
Regaud	3.8	1.24	0.69	0.55	.94	.64	.30
Altmann	3.9	2.09	0.60	1.49	1.55	.59	.96
Formol–Müller	4.2	1.19	0.69	0.5	.99	.65	.34
Formol–alcohol	5.2	1.24	0.79	0.45	.66	1.03	— .37

COMMENTS

Although the experiments here reported do not simulate the conditions exactly occurring in ordinary fixation, it is felt that they give basic information regarding the precipitating potentialities of some of the fixing fluids and combinations. Each of the solutions tested consistently affects the emulsions of liver and brain in a manner which produces a characteristic type of precipitate with a rather constant vol-

ume. Fundamentally, the investigation indicates that the type of tissue, the hydrogen-ion concentration and the chemical formulae of the fixing solutions are all important.

In respect to the type of tissue, quantitative differences are always obtained between the liver and brain emulsions subjected to identical fixing solutions indicating that the chemical composition of the tissue is a factor.

Maximal precipitation of proteins is obtained in the buffered solutions at certain hydrogen-ion concentrations. This gives possible information regarding the iso-electric point of the proteins of brain and liver. The greatest amounts in brain emulsions from the quantitative viewpoint take place in the pH range between 0.9 and 1.5 whereas in the liver experiments it occurs between 3.0 and 4.0. It appears that varying the pH in the buffered solution alters the quantity of the precipitate but not its character as far as can be judged by gross examination. We do not know whether the staining characteristics might be changed under ordinary conditions of fixation from these fixing solutions following alterations in hydrogen-ion concentration.

That the chemistry of the fixing fluids, outside of their pH factors, is also of significance is indicated by the quantity, the character and the specific gravities of the precipitates in comparison with those in the buffered solutions. With the exceptions of certain of the acid fixing solutions (acetic, nitric and osmic), the differences are pronounced between the two. In some instances, the quantity of the precipitate will be greater than that in the corresponding buffer solutions and in others less. This should be due to chemical factors in the fixing fluids.

From the experiments, it is found that the precipitating action of any fixative may reflect that of the primary solutions contained in it. Fixatives which are made up of fixing fluids which singly produce relatively heavy precipitates will likewise cause larger quantitative effects.

Although it is undoubtedly of importance, the question as to the chemical composition of the precipitates formed in the foregoing experiments is felt to be beyond the scope of the investigation.

CONCLUSIONS

The precipitating action of 10 primary fixing solutions and 10 commonly used fixing combinations as well as buffered solutions of variable pH's has been investigated on both liver and brain emulsions. The results indicate that the type of tissue, the hydrogen-ion concentration and the chemical formulae of the fixing solutions each play a role in fixation. This can be shown quantitatively and by the character and specific gravity of the precipitates. Maximal precipitations in the buffered solutions occur between the pH's of 0.9 and 1.5 in the brain experiments and between 3.0 and 4.0 in the liver series which suggests that their iso-electric points may be in these ranges. That both the type of tissue and the chemical composition of the fixing fluids are of importance can be shown, first, by the quantitative differences obtained between the two types of tissue with any one fixing solution and, second, by the fact that the various fixing fluids tested do not give identical values on one type of emulsion. The precipitating action of any fixing combination also seems to be a quantitative and qualitative reflection of the basic fixing solutions of which it is composed.

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SECTIONING THE HUMAN BRAIN WITH REFERENCE TO THE FRANKFURT PLANE

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FIVE FIGURES [1]

Three or four years ago the senior author advised the departmental technician, Ralph Bouldin, to construct a simple "macrotome" for cutting the fixed and hardened human brain evenly and smoothly into serial slices of measured and uniform thickness. With the help of an instrument maker, Mr. William Holzheimer, he made such an apparatus and described it in "Science" of November, 1947 (vol. 106, p. 527), though no special originality was claimed for it, since "miter-box" devices of various kinds have been used before by neurologists to guide the brain-knife.

The usual method of making coronal or frontal slices of the preserved brain, removed from the cranium, is to draw the knife through it at right angles to its mid-sagittal plane and perpendicular to the base on which it "found" its position of rest. Such sections represent planes approximately transverse to Forel's axis, which is defined by a line connecting the occipital and frontal poles of the same cerebral hemisphere. Although cross-sections prepared in this way are satisfactory for general or casual examination in the laboratory, a more definite or universally acceptable standard of reference or orientation, namely the Frankfurt horizontal plane of the head, would serve more adequately the demands of the anthropologist, anatomist, pathologist, experimental neurologist and brain surgeon. Accordingly, we set out to

modify the macrotome so that the brain could be cut into coronal slices which would be perpendicular to that plane, or into horizontal slices parallel with it.

Fortunately, a staff member, the neurologist Gerhardt von Bonin, had published a paper ("The axis of the forebrain in macaque and man") several years previously ('42; Am. J. Phys. Anthrop., vol. 29) in which he gave the results of a roentgenographic study — of 114 human adults (78 males and

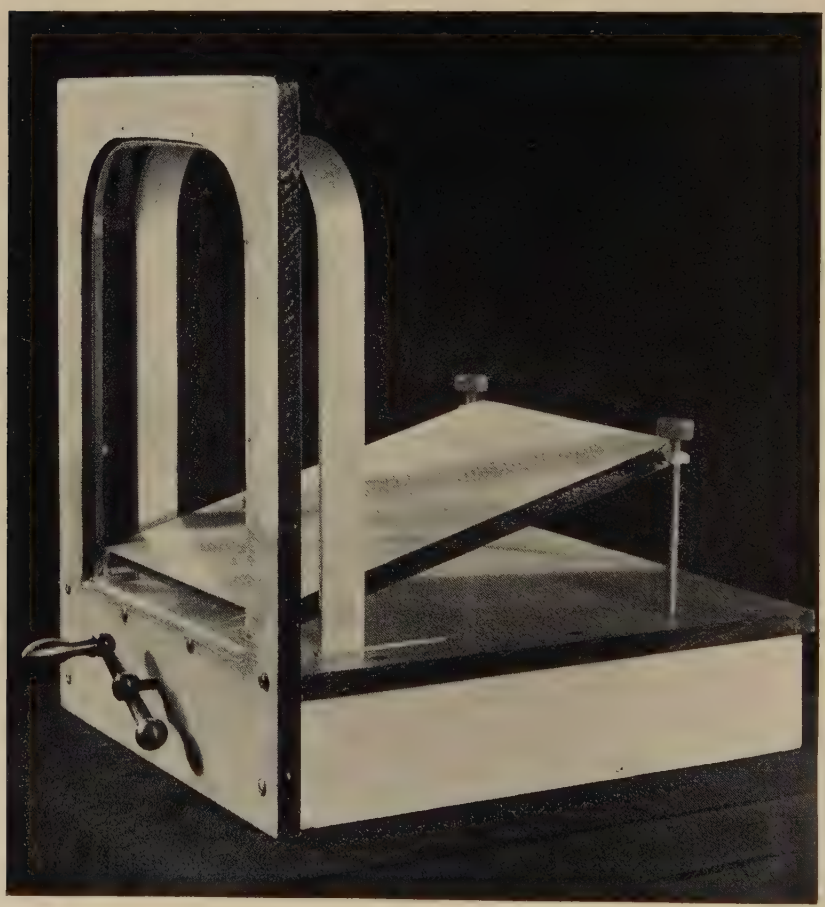

Fig. 1 Macrotome with adjustable platform for tilting and slicing the brain with reference to the Frankfurt plane.

36 females) and of 49 macaques — that determined the angle between the cerebral occipito-frontal axis (of Forel) and the Frankfurt cranial horizontal plane. He found this angle to be 10° (actually 9.8°) in the human and 7.5° in the macaque.

The macrotome, pictured in figure 1, is adapted to these findings. Since the plan of its construction is depicted sufficiently clearly in the photograph, and its dimensions are

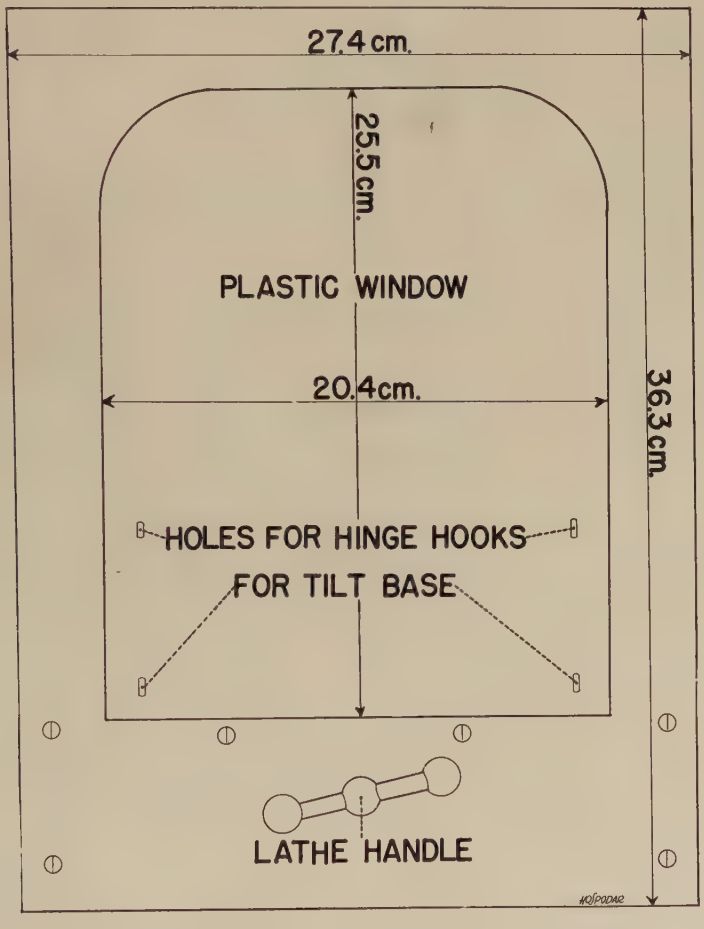

Fig. 2 Plane of construction of the macrotome in front view. (The dimensions of height, width and length of the apparatus indicated in this and the following two drawings could probably be reduced with advantage.)

indicated in the drawings (figs. 2–4), we can dispense with a detailed description of it. It is to be noted that the vertical front is supplied with a window of plastic ("Plexiglas," 6.5 mm thick) which permits the observer to see if the cut surface of the brain rests flush against it before the next slice is made. The thick sheet of plastic is held in place by an arched metal flange fastened to the back of the vertical board by means of counter-sunk screws, thus making pos-

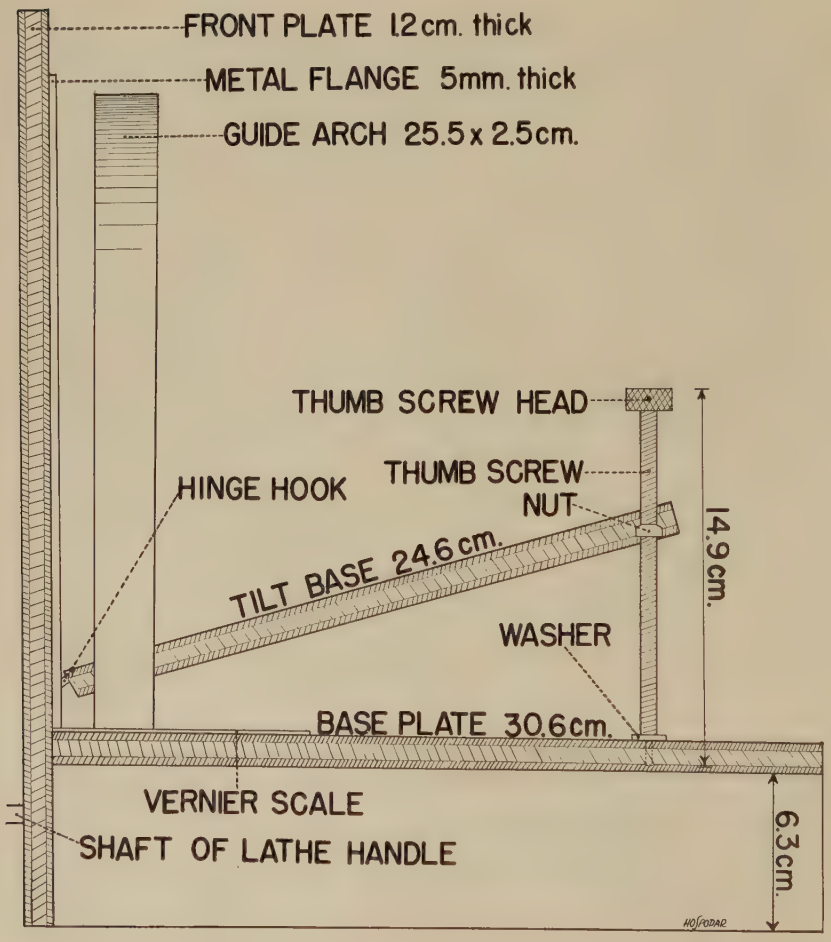

Fig. 3 Plan of construction of the macrotome in profile view.

sible the easy removal and replacement of the "Plexiglas" if necessary.

Since this metal flange is 5 mm thick, the thinnest brain slice which can be cut with our macrotome is somewhat more than that, if the arched metal knife-guide is moved as close to the flange as the thickness of the knife-blade will allow. In this position the sections cut by us measure about 7.5 mm

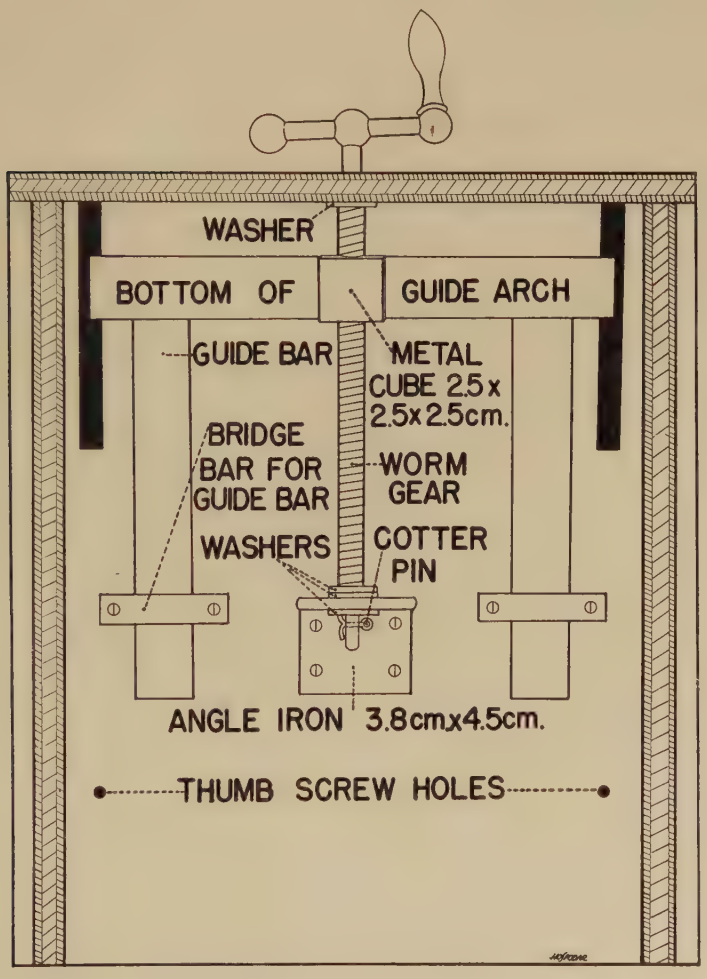

Fig. 4 Plan of construction of the macrotome seen from below.

in thickness, so that an adult human brain of average size will yield 16 to 18 serial coronal slices or, if cut horizontally, 14 to 16 slices. Thicker slices can be cut, of course, by moving the knife-guide away, that is, back by means of the worm-gear; a vernier on the horizontal base of the macrotome shows the thickness.

The first model of our macrotome was made of steel plate (5 mm) and it was not provided with a window. We found this apparatus to be too cumbersome. The second model, illustrated here, is built of three-ply (hard maple) plywood, except for its metal attachments, and it has been coated several times with sprayed plastic. Though this model has proved to be entirely serviceable, long usage may demand greater rigidity and durability. A macrotome made of a light metal, like aluminum, should be a satisfactory one, if the expense of construction is not a factor to be considered.

An essential feature of the macrotome is the adjustable platform on which the brain rests and which can be inclined up or down to the desired angle (fig. 3). Two pairs of holes in the plastic window (fig. 2) for hinging or pivoting the platform at its front edge, and a pair of long thumb screws at its rear to adjust the degree of tilting, make possible either an upward or a downward tilt according to which end of the brain will come first in the serial slicing. By tilting the platform until Forel's axis of the brain corresponds with the stem of a bevel- or an angle-square, set at 10° off 90° (fig. 5), while the body of the square rests against the squared edge of the macrotome, the brain is orientated accurately relative to the Frankfurt plane.[1]

For coronal slicing of the brain, beginning with the occipital pole, the platform or tilt-base is hinged in the lower

[1] Originally we had planned to attach a protractor scale to the side of the macrotome, but for obvious reasons such a device would have been less simple and less convenient for both coronal and horizontal slicing of the brain than the application of the angle-square. For this suggestion we are indebted to our colleague, James E. Davis, Assistant Professor of Mathematics in the College of Pharmacy.

holes of the window and is inclined upwards. If the coronal slicing is to begin with the frontal pole, then the tilt-base is hinged in the upper holes of the window and is inclined downwards.

For horizontal slicing — that is, parallel to the Frankfurt plane — the brain is set on end, leaning against the window, and is adjusted in position until Forel's axis conforms to the angle-square properly held to the horizontal base of the macrotome.

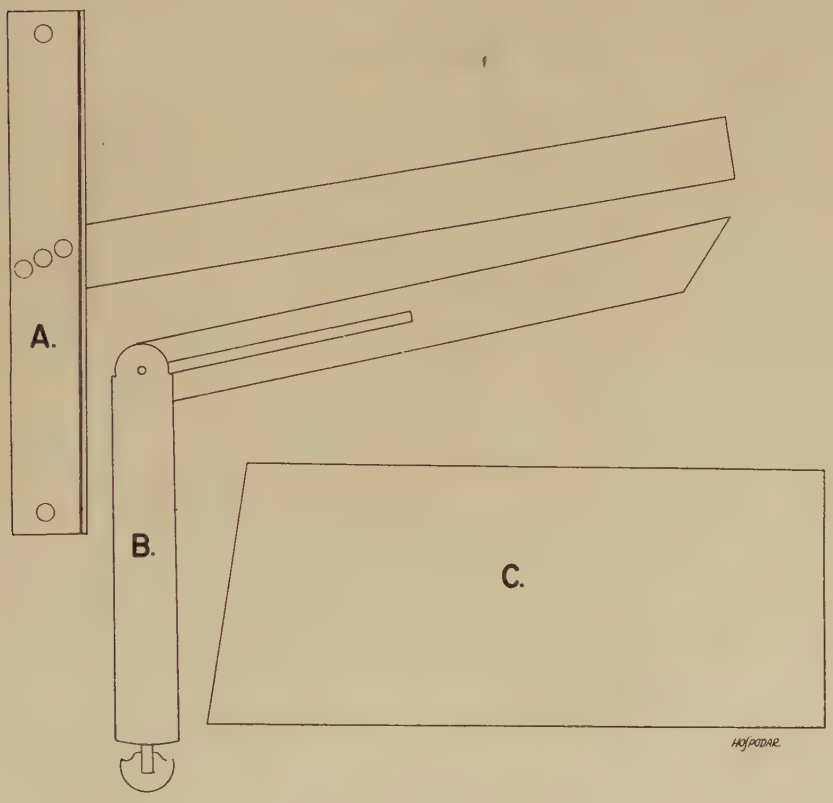

Fig. 5 Simple types of angle-squares, set at 10° off 90°; *A*, metal, with fixed stem; *B*, a carpenter's metal bevel-square with adjustable blade; *C*, a wooden board properly cut and squared. (The blade or edge along which the sighting is done must be appreciably longer than Forel's axis of the brain; one, 25 cm long is adequate.)

If, when the first slice is being cut, the mid-sagittal plane (great sagittal fissure) of the brain stands at right angles to the macrotome's window, all subsequent slices are uniformly parallel to each other provided the cut surface of the brain is held always flush against the surface of the window.

Difficulties will be encountered, of course, in holding the cerebral hemispheres, as well as the cerebellum, together as the slicing approaches the ends or poles. Gelatine or agar-agar can be used to serve as binding material, but this requires to be removed from the peripheries of the slices later before gross-staining is done. Generally the employment of embedding media is not necessary if two operators work together, one pair of hands holding together different parts of the brain and the other pair manipulating the brain-knife.

Needless to say, to prepare slices of even thickness and clean-cut, that is, free from rippling or knife marks, it is essential that the brain is well-fixed and formalin-hardened, the knife smooth-edged and sharp, and its blade of sufficient length to permit a long, gliding stroke from hilt to tip.[2] To overcome the tendency of a long blade to bow from side to side, it must be kept firmly against the knife guide, one hand of the operator holding it to the farther limb of the guide, and the other hand to the nearer limb as it draws the blade smoothly, unhesitatingly and with unchanging force and direction through the mass of the brain.

Methods of staining the serial brain slices and their embedding as wet specimens in transparent plastic for teaching or museum preparations are described in another paper that is being prepared for publication.

[2] The brain knife we have used for 15 years has a blade 50 cm long, 2.7 cm wide, and 2 mm thick at its back edge (measured near the 15 cm handle). We obtained this knife from a wholesale company dealing in restaurant equipment.

ELECTRON MICROSCOPE INVESTIGATION OF THE STRIATED BORDER OF INTESTINAL EPITHELIUM

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EIGHT FIGURES

The constant occurrence of a striated border on the free surface of the cells lining the vertebrate intestine suggests that the fine structure of this border may be of functional significance. From this standpoint as well as from a purely morphological one, the border is of interest, yet more than 100 years of observation have failed to clarify its structure with certainty. Since our continued ignorance has apparently been due to the limitations of the light microscope, the present study with the electron microscope was undertaken by the use of the thin tissue sectioning techniques developed by Pease and Baker ('48).

As long ago as 1841, attention was first called to the existence of the border by a drawing in Henle's *Allgemeine Anatomie*. Here the free surface of the intestinal cells of the rabbit was depicted as a clear, homogeneous, and apparently structureless layer. Some 15 years later the vertically striated character of the border was recognized almost simultaneously by Funke (1856), Koelliker (1856, cited in Patzelt, '36) and Welcker (1857). The question of interpreting these striations immediately presented itself. Funke (1856), describing the striations in the intestinal epithelium of the rabbit, noted their strong resemblance to cilia, but because of the lack of any observable motility denied their identity with true cilia. He suggested the possibility that the stripes might be the

expression of fine, cylindrical pore-canals which could provide paths for absorption of fats. Koelliker (1856, cited in Welcker, 1857 and Baker, '42) studying the striations in mammals, a bird, and several amphibia, found that their fineness prohibited an exact decision, but favored the pore canal interpretation. Welcker (1857), however, stated that critical focusing upon the border of the dog left no doubt as to the existence of actual pore canals.

Soon an alternative interpretation was presented by Brettauer and Steinach (1857). Careful observation of the border in rabbits, guinea pigs, dogs and one human, convinced these investigators that the striations were not the optical expression of pore canals, but of very small, individual, rod-like structures ("Stäbchen"), vertically oriented and standing close beside one another. Thus from early times there existed two opposing views on the fundamental composition of the border. By some it was considered to be a surface layer pierced by cylindrical canals, by others an aggregate of small rod-like projections.

Subsequently many microscopists of the late 19th and early 20th centuries endeavored by more careful techniques and searching observations to demonstrate more exactly the composition of this border. For a complete compilation of these reports the reader is referred to Patzelt's chapter on the intestine in Von Möllendorff's *Handbuch der mikroskopischen Anatomie des Menschen* ('36), and to Baker's ('42) more detailed discussion of certain of these papers.

The tenor of these accumulated findings was such that the rod theory came to be much more widely accepted than the canal theory. Probably the most elaborate, though by no means entirely original, conception of the structure of a rodded border was that presented in the papers of Zipkin ('04) on the monkey, and Clara ('26) on the bird. They confirmed previous reports of a two-layered appearance of the border due to the division of each rod into an inner, shorter, more deeply staining portion (Innengleid) and an outer, longer, more lightly staining portion (Aussengleid). They also described

two sorts of granules occurring in connection with the rods: a fairly large, ellipsoidal granule, darkly stained with iron hematoxylin, situated directly under the cell membrane at the base of each rod; and a smaller, round granule at the junction of the inner with the outer part of each rod. Clara ('26) figured a ground substance throughout the border occupying all the spaces between the rods, and drawn into a sleeve-like mantle over the inner rod parts. Zipkin ('04) found only inconclusive evidence of ground substance in the inner layer of the border, and apparently believed it to be absent in the outer layer.

These views illustrate the general trend of opinion on border structure previous to 1942. However, brief mention of some of the more outstanding divergent reports is pertinent. Zimmermann (1898), in his examination of the intestine of an executed criminal, found that the epithelial cells bore not only a two-layered, rodded border, but also longer protoplasmic processes passing out between and beyond the rods. In contrast to the picture of a more or less static arrangement of rods, R. Heidenhain (1888) conceived of a mobile, changing border composed of protoplasmic extensions of the cell which could be extended to varying distances or withdrawn entirely. As late as 1931, Thanhoffer, on the basis of microdissection experiments with the border of the frog, stated that both canals and rods were entirely lacking and that the striations were an optical, rather than a morphological property. Thus, while the rod theory of border structure received the most general acceptance, conflicting views failed to be reconciled.

This lack of real agreement persisted until 1942 when John R. Baker reexamined the question. Approaching the problem systematically, he employed a variety of animals (newts, frog, slow worm, starling and rabbit) and a number of different fixatives and staining procedures. Instead of diagrams, he presented for the first time actual photomicrographs of the border. His observations, carefully made at the upper limits of the light microscope, led him to refute the rod theory and to conclude that the border indeed consisted of a continuous

layer of material perforated by spindle-shaped canals. It appears that Baker's findings are as sound as any which could be made with modern light microscopy. Nevertheless, the very fineness of the structures still suggested (as Koelliker said in 1856) that an unequivocal picture could not be obtained with the light microscope. Therefore the present study of the border with the electron microscope was begun. Preliminary results were reported by the authors in 1949.

MATERIALS AND METHODS

The small intestine, usually the ileum, of normal, young, adult rats was fixed by perfusion with a Zenker-formol-osmic acid combination, or by immersion in 2% osmic acid. Very small pieces of tissue, not more than about 2 mm across, were doubly imbedded in a 12% solution of Parlodion and hard paraffin (M.P., 65°C.).

Sections from 0.05 to 0.2 μ thick were prepared and mounted on screens for electron microscopy by methods essentially like those first described by Pease and Baker ('48). As the work progressed, modifications of the technique were employed (Baker and Pease, '49). Sections were flattened by floating them on dioxane (or 40% ethyl alcohol) which was then allowed to evaporate. After removal of the paraffin with benzene, the slide was immersed in an 0.2% Parlodion solution. Both slide and sections were thus coated with a thin Parlodion film, which was dried in air. Screens were then placed over each section and the whole film floated off on water. Screens with sections adhering to them could then be picked up, and were ready for use.

Sections thus supported by a thin Parlodion film were studied and photographed with the electron microscope at magnifications of 800 to 7000 times; electron micrographs were enlarged up to 5 times. To increase image contrast, an aperture 0.001 inch in diameter was used in the objective lens for some of the later micrographs.

For comparative studies, sections of the same blocks were also cut at 0.5, 1.0, 2.0, and 5.0 μ, stained in iron-hematoxylin, and examined with the light microscope at magnifications up to 2000 times.

OBSERVATIONS

A. Studies with the light microscope

The appearance of the striated border of the rat in conventionally cut sections stained with iron-hematoxylin and eosin is shown in the inserts on figures 1 and 2.

1. *Striations.* In both inserts the reality of the vertical striations is obvious. Nevertheless it is apparent that they defy resolution into either rods or canals. If one considers particular regions of these figures, however, the basis for some of the conclusions of previous investigators becomes understandable. In some areas the striations are sufficiently coarse (insert, fig. 2) to tempt one to the conclusion that the relatively dark lines might be rods, or that the light stripes might be canals. More often, however, the striations are much finer (insert, fig. 1) and it is impossible to differentiate one stripe from another. While it is true that photographs, especially when reproduced, do not do justice to this material, it must be emphasized that not even first hand microscopic observations are adequate to reveal the nature of the vertical striations.

2. *Granules.* Wherever the border has been cut vertically a prominent line, darkly stained with iron-hematoxylin is seen at the base of the striations. In some places this line appears homogeneous and structureless (insert, fig. 1). In other places (insert, fig. 2) close scrutiny reveals that it is not quite uniform in density and thickness, and direct observation with the microscope suggests that it might be made up of individual granules. These, if they do exist, are the "Basalellipsoide" described so often in the literature.

Very occasionally areas were encountered in this stained material which showed a very fine, pale line, running parallel but peripheral to the prominent dark line. This is apparently

the line which was described by many as producing the division of the border into the wider, outer layer and narrower, inner layer. Zipkin ('04) and Clara ('26) suspected that it represented the sum of the smaller, round "limiting" granules which they believed existed in each rod at the junction of its outer and inner parts. In our material, even the line itself, let alone the granules, is at best inconstant, and only barely visible.

It may be concluded, finally, that while stained sections of the striated border of the rat show the same features which have served as a basis for other descriptions of the border, light microscopy has not afforded a conclusive answer on its fine structure.

B. Electron microscopy

1. *Fundamental structure of the border.* In figures 1 and 2 the striated border is shown at magnifications of about 22,000 times. For comparison, the small inserts show the border as it appears with light microscopy at 2000 times. The electron micrographs (EMGs) clearly demonstrate that the border is composed of separate, perpendicular processes. These units are far more minute in size than any conceived by earlier advocates of the "rod" theory. Figure 1 presents a clear impression of individual processes; yet even in this section only 0.1 μ thick, they are not in a single layer but stand behind each other at least two deep. This fact attests to their small caliber. Figure 2 shows a section which is of special value because of its extreme thinness, although unfortunately it has suffered some stretching and tearing. Here the processes are in a single layer, and their individuality is accordingly more apparent.

Cross sections of the processes are shown where the border has been cut obliquely (figs. 3 and 5). Such views give a clear idea of the small diameter of these structures, of their individuality, and of their arrangement. These figures also dispel any lingering notion that the border is a continuous

layer of material perforated by canals. The small units of tissue do not bear the kind of spatial relationship to the light areas between that would have to exist if the canal theory were valid; the light spaces are not delimited by the solid tissue as canals would necessarily be delimited.

That such processes are the structural units of the border is further demonstrated by figure 8. Here, because their usual regular arrangement has been greatly distorted, single processes are seen with great clarity, widely separated from their neighbors.

2. *Form and dimensions of the processes.* A study of figures 1–3 leads to the conclusion that these processes are columnar, with blunt, slightly rounded tips. Some variability in their dimensions is apparent. The most frequently encountered proportions are seen in figure 1. Unusually long, slender processes (fig. 2) and especially short, plump ones have been observed.

Actual measurements of the dimensions of the processes have been made on a large number of EMGs. Measurements of length were made only for that part of the process which projects freely into the lumen. This length ranges from 0.26 μ to 1.0 μ, and averages 0.62 μ. The diameter ranges from 0.04 μ to 0.1 μ, and averages 0.08 μ. In general, the diameter tends to vary inversely with the length. Thus a process 0.9 μ long (fig. 2) is 0.04 μ in diameter, whereas a process only 0.37 μ long is 0.09 μ in diameter.

3. *Arrangement and numbers of processes.* It has already been pointed out that the processes are so closely packed that only in the thinnest vertical sections do they appear in a single row, even though none of the sections exceeded 0.2 μ in thickness. Additional evidence of their close packing is provided by those views which show the processes cut in cross section. Here (figs. 3 and 5) many actually make contact with their neighbors, and others are separated by spaces far smaller than their own diameters. In addition, figure 3 strongly suggests that the processes are arranged in fairly straight successive rows. Also in this figure an interesting view of the sides of

the processes in one such row is exposed by an oblique cut and bending of the processes.

No entirely accurate estimate of the numbers of processes present can be made from the data at hand. However, since it is evident that extremely close packing is the characteristic arrangement, it is possible to calculate the maximum number which could be borne by a single cell. On an average, the cross-sectional area of one process is 0.005 μ^2 and that of the free surface of one cell is 14.2 μ^2. Therefore, if the packing were infinitely close, a single cell could support about 3000 processes and a square millimeter of intestinal surface could support approximately 200,000,000 (2×10^8). However, since very small spaces undoubtedly exist between neighboring processes, these figures must exceed somewhat the numbers actually present.

In an alternative method for calculating the number of processes per unit area, an actual count is made of the number occurring per unit length of border on EMGs of thin vertical sections where the processes appear in a single layer. Then, if one assumes a uniform distribution throughout the border, the number per unit area is easily computed. A count made on figure 2, for example, gives a value of 190,000,000 (1.9×10^8) per square millimeter which is in good agreement with the value obtained above.

4. *Relations of the processes to the cell.* In low power EMGs ($\times$ 4000) two horizontal lines appear with great regularity at the junction of the cell proper with the base of the border. Certain high power EMGs, especially those taken with an aperture in the microscope, clarify these relationships to some degree. In figure 4 the two lines are very apparent. Here the processes clearly originate beneath the superficial line and extend out beyond it into the lumen. The actual points of origin of the processes are not well defined, but, as nearly as can be determined, seem to coincide with the deeper of the two lines.

There is no real proof as to which line represents the outer membrane of the cell proper. However, several observations indicate that the superficial line marks the cell limits. If fig-

ure 6 is carefully examined the superficial line appears to pass behind some of the processes, in front of others, and between adjacent ones. Therefore, it apparently does not represent a differentiation of the processes themselves, but rather a membrane between the processes.

The deeper line, in contrast, is generally thicker than the superficial one, and less regular in contour. In many views successive round swellings can be seen along its length (figs. 4, 6 and 7). These swellings are best defined in figure 7, yet it is obvious that even here they are not shown with sufficient clarity to warrant further conclusions as to their nature. Likewise the relationship of the deeper line to the bases of the processes remains in considerable doubt. Certain of the swellings seem to lie at the bases of particular processes, but whether this is the fundamental arrangement cannot be determined. Finally, the general appearance of the deep line suggests that it is a differentiated constituent of the cytoplasm, rather than the limiting membrane of the cell.

In conclusion, it is probable that the superficial line is the cell membrane. This hypothesis is further supported by the fact that sections of terminal bars, easily identifiable in low power EMGs, extend peripherally beyond the deep line to the level of the superficial one.

5. *Character of the individual processes.* In general the processes as seen in these EMGs are homogeneous from base to tip. The only clue as to their internal structure is derived from the views of separated, tangled processes in figure 8. Clearly they are not homogeneous. On the contrary there is some differentiation within them which is suggestive of horizontal striping or possibly of some spiral pattern.

DISCUSSION

The exceeding smallness of the processes is apparent from the foregoing observations. Especially noteworthy is the fact that their diameters are in nearly all cases less than 0.1 μ. It is clear that the individual structures composing the striated border have never actually been resolved by light microscopy,

and that the electron microscope has afforded the first clear view of them.

However, these EMGs demonstrate that in the fixed state the processes of the border are not always perfectly straight and equidistant from each other, from base to tip. They sometimes appear bent, so that some lean toward each other and others away (figs. 2 and 6). Such clumping together of several processes must account for the illusion of separate rods in the light microscope. This is borne out by the fact that early workers (Patzelt, '36) estimated the diameter of the rods to be about 0.5 μ as compared with our average figure of 0.08 μ, and the number per cell to be only 41 (Zipkin, '04) in contrast to a count of about 3000 indicated by electron microscopy. Similarly, the spaces produced by the occasional separation of two adjacent processes must have been the basis for the canal interpretation. The canals described by Baker ('42) as being sometimes as wide as 0.5 μ probably correspond with spaces of this sort, certainly not to the regular spaces between upright processes which EMGs have shown to be smaller than the processes themselves.

There are two interesting questions still to be answered in regard to the structure of the border. First, where are the limits of the cell? As previously stated, evidence of these studies suggests that the cell membrane lies just peripheral to the basal ends of the processes. The processes then would have an intracellular origin. However, even if this assumption is correct, there remains the problem of whether the processes penetrate the cell membrane or whether extensions of the membrane are carried out over each projection. In the latter case the whole length of the process would be essentially a continuation of cell protoplasm and not truly extracellular in any sense. The EMGs taken with an objective aperture often show the basal end of the process to be more slender than the portion beyond the membrane (fig. 4). Such a situation lends itself to this concept of the cell membrane being carried out to envelop the peripheral part of the process. However, no such membrane is actually distinguishable on the processes in

any pictures thus far obtained. It is therefore impossible to draw any conclusions in this regard from present evidence.

It may be recalled that Zipkin ('04) and Clara ('26) figured the cell membrane as lying just peripheral to their basal granules. Therefore the border proper was thought to include the entire length of each rod, with its shorter, darkly staining, inner portion, and its longer, lightly staining, outer portion. On each rod a small round limiting granule marked the junction of inner and outer parts. The EMGs offer no confirmation for this concept of a limiting granule as an integral part of each rod. However, it seems probable that the line considered as the sum of these limiting granules by Zipkin and Clara, and faintly visible in some of our stained material, actually is the superficial membrane identifiable in the EMGs. If so, the portion of the process beneath this membrane would correspond to the traditional short, inner part of the rod; that portion projecting beyond the cell surface, to the long outer part of the rod. Nevertheless the EMGs do not corroborate the idea of structural differentiation between these parts of the processes; rather they suggest that each process is uniform in character throughout its length.

The second problem concerns the exact manner of origin of the processes. What is their relation to the deeper horizontal line and the swellings seen upon it? Although in the literature there are repeated descriptions of separate basal granules in this location (Patzelt, '36) EMGs have not thus far demonstrated such granules clearly. Interestingly enough, Baker ('42) stated that there seemed to be a diffuse substance lying between the so-called basal granules which gave the same staining and histochemical reactions as the granules themselves. Our pictures showing swellings occurring along the length of a more or less continuous line (figs. 4 and 7) suggest that Baker's interpretation might be correct. Whether there is one swelling or granule for each individual process is still undetermined. If such granules do occur, they must

be much smaller than earlier observations indicated. Final solution of these relationships certainly awaits further investigation.

At this time one can only guess at the functional significance of these minute processes constituting the border. It seems very likely that they play some active role in the absorptive process. EMGs have shown that the processes vary in form, some being long and slender, others shorter and more plump. These deviations from the mean were encountered in the intestine of healthy, young adult rats from a stock colony maintained on a normal diet. Some of the variations in their length should probably be attributed to the location of the cells supporting them, for it is well known that the striated border is widest on the tips of the villi and narrows down progressively along the sides of the crypts. However, the pronounced tendency for the length of the processes to vary inversely with their diameter, suggests that variations in form do occur regardless of position. It is unlikely that the shorter processes in the crypts are necessarily all more plump. It is therefore tempting to speculate whether these changes in form may not well reflect different functional states.

In this connection it is pertinent to recall that Brettauer and Steinach (1857) compared the appearance of the border in starved and in well nourished animals. After subjecting guinea pigs, rabbits, and dogs to periods of 20–36 hours without food, they found that the border as a whole was relatively broad and very plainly striped. It was in such borders especially that they believed that they could see individual rods or Stäbchen. By contrast, the borders in well fed animals generally were much narrower and failed to show plain striations. No data of this sort have yet been secured with the electron microscope, but such an investigation may prove feasible in the future.

The complete explanation of the role of the processes in absorption remains to be found. Nevertheless, it is very probable that to a large extent the significance of the processes lies in the fact that they afford a considerable increase in

absorptive area. The free surface area of one process can be calculated from measurements of their length and diameter to be 0.155 μ^2. The number of processes present on one square millimeter of surface has been estimated at about 200,000,000 (2×10^8). Therefore the processes of the border must provide an increase in surface of about 30 times. The concept that such an increase in absorptive area has significance is entirely acceptable in view of the other familiar features of the intestinal wall: the coiling, the folds of the mucosa, and the villi.

SUMMARY AND CONCLUSIONS

1. Very thin sections (0.05 to 0.2 μ) of the intestinal epithelium of the normal, adult rat have been studied with the electron microscope at magnifications up to 35,000 times.

2. Electron micrographs demonstrate that the striated border on the free surface of the absorptive cells is composed of very minute processes projecting vertically into the lumen.

3. These processes are columnar in form, with rather blunt, rounded tips.

4. The average length of the free part of the process is 0.62 μ; the average diameter is 0.08 μ. From these dimensions it is clear that the individual processes could not have been seen with the light microscope.

5. These processes are packed very closely together and are present in great numbers. From the data at hand it has been calculated that a single cell must bear nearly 3000 processes and a square millimeter of intestinal surface, some 200,000,000. Therefore, though the individual processes are very small, their combined numbers afford an apparently significant incease in the absorptive surface of the intestine. This increase has been estimated to be about 30 times.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Electron micrographs of the striated border of the small intestine of the rat.

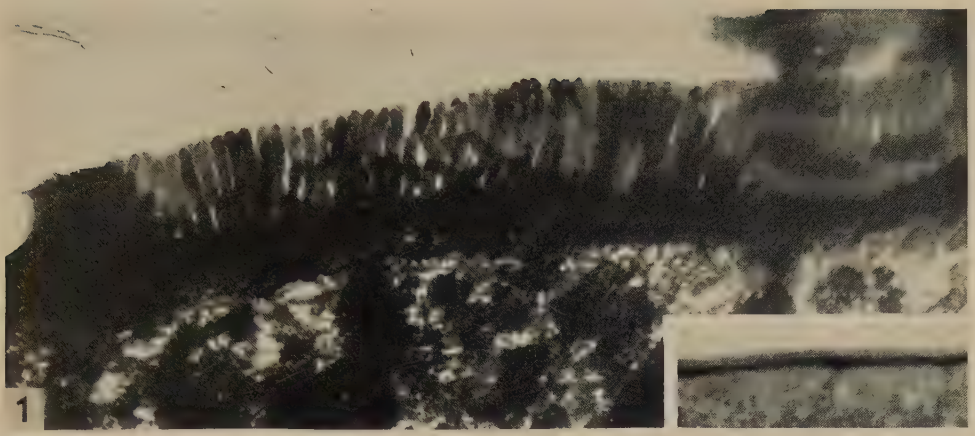
1 Vertical section showing minute, individual processes which constitute the border. Processes here are of average length and diameter. Osmic acid fixation. × 21,600.

Small insert shows comparable strip of border as it appears with the light microscope. × 2000.

2 An unusually thin, vertical section showing only a single layer of processes. Note that these processes are longer and more slender than those of figure 1. Holes where processes are pulled widely apart are artifacts due to the stretching and tearing of the section evidenced by the breaks in the supportive Parlodion membrane. Zenker-formol-osmic acid fixation. × 22,600.

Insert is a photomicrograph of a similar area where the border is relatively high.

3 Oblique section of the border showing many of the processes cut transversely. Note their small diameters and close arrangement. In the lower left corner, the sides of the processes have been revealed. Osmic acid fixation. × 21,600.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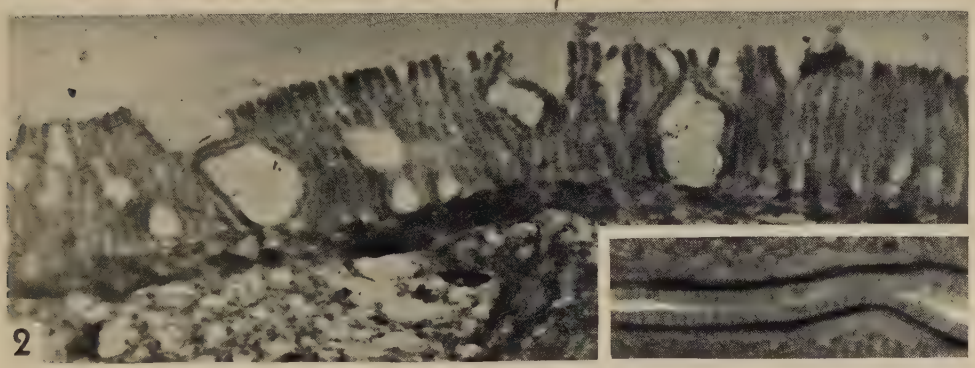
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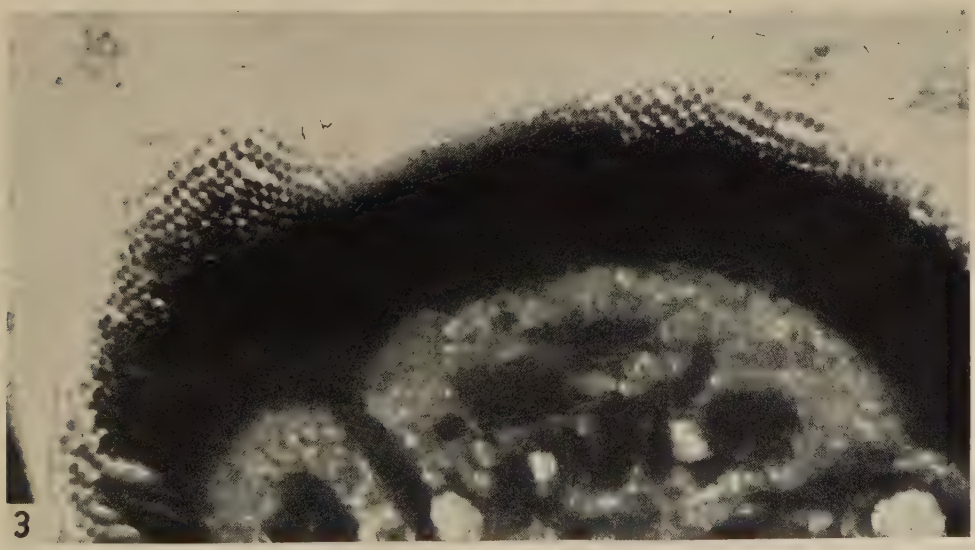
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PLATE 2

EXPLANATION OF FIGURES

Electron micrographs of the striated border of the small intestine of the rat. Osmic acid fixation.

4 Vertical section showing the two horizontal lines at the base of the border. The processes originate in the region of the deeper, heavier line, and pass through and beyond the more delicate superficial line. Successive swellings may be seen along the deeper line. × 19,000.

5 Oblique section showing some processes cut transversely. Observe that the processes are extremely close together and only very small spaces remain between them. × 27,360.

6 Vertical section in which the superficial horizontal line (cell membrane ?) can be seen passing in front of some processes, behind some, and between others (right arrow). Swellings on deeper line may also be seen (left arrow). × 19,000.

7 Vertical section in which swellings along the deeper horizontal line are relatively distinct. × 17,100.

8 A region where the orderly arrangement of the processes of the border has been distorted, probably in the preparation of the tissue. The result is an advantageous view of individual processes, well separated from their neighbors. Some internal differentiation is apparent in the processes here. × 32,500.

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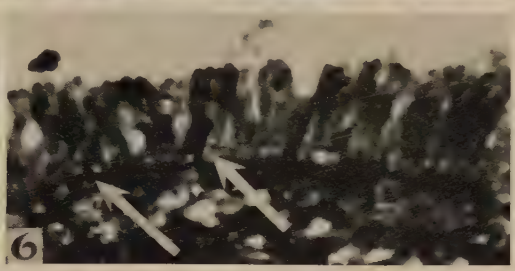
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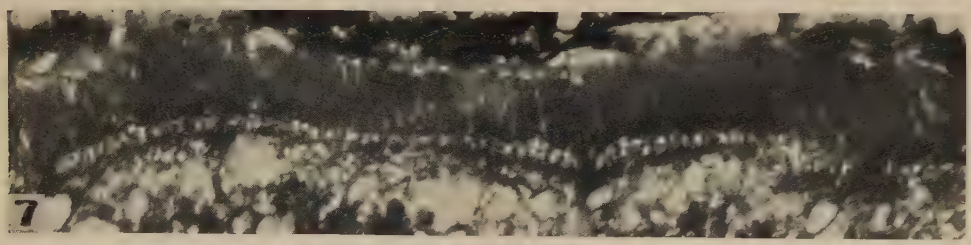
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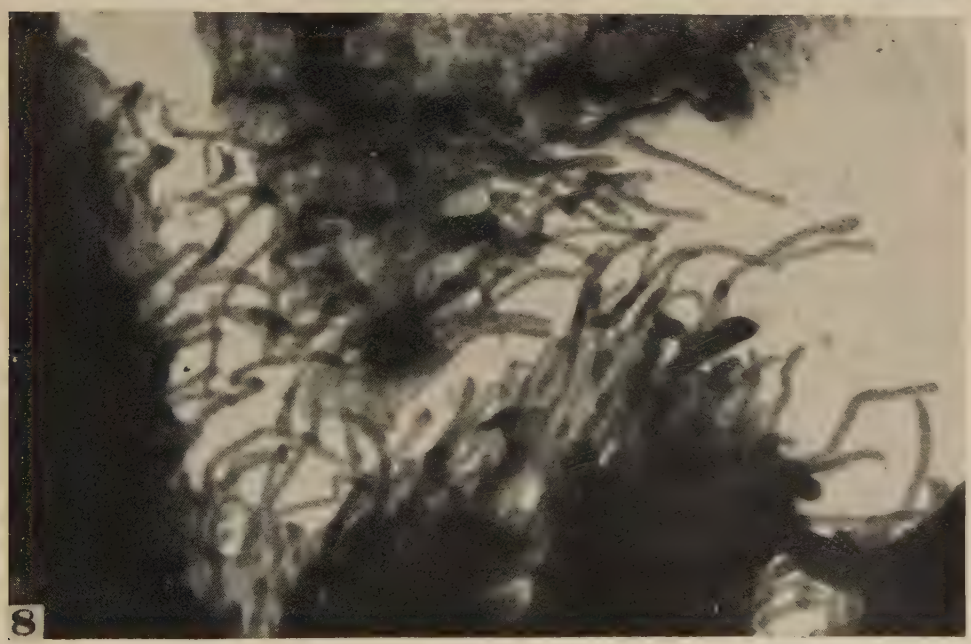
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A NEW TECHNIQUE FOR RAPID FREEZING AND DEHYDRATION OF TISSUES FOR HISTOLOGY AND HISTOCHEMISTRY

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ONE FIGURE

Histological and histochemical investigations have been hampered by the problem of fixation procedures and the artifacts which they introduce. The artifacts and distortions of tissues that are produced by most methods of fixation can be minimized by use of the freezing-drying method of preparing tissues (Altmann, 1890; Gersh, '32). In principle this method consists of rapid freezing of tissues at low temperatures, followed by dehydration *in vacuo* of the frozen tissues. It has been shown by Flosdorf and Mudd ('35, '38) and by Elser, Thomas, and Steffen ('35) that there is little or no change in the chemical nature and biological activity of sera, enzymes and biochemicals after treatment in this fashion. Following dehydration, the dried tissues may be subjected to histochemical procedures, or may be embedded directly in paraffin or celloidin for use in histological studies.

The apparatus and technique introduced by Gersh ('32) have been subjected to a number of modifications (Hoerr, '38; Simpson, '41; Taylor, '44; Wang and Grossman, '49) and have been widely used in histological research. It seems that certain further advantages would accrue if (1) the apparatus were made of metal instead of the more fragile glass, (2) the procedures were simplified and standardized, (3) the apparatus were less expensive and easier to operate, (4) the dehydration were accomplished in less time, and (5) the

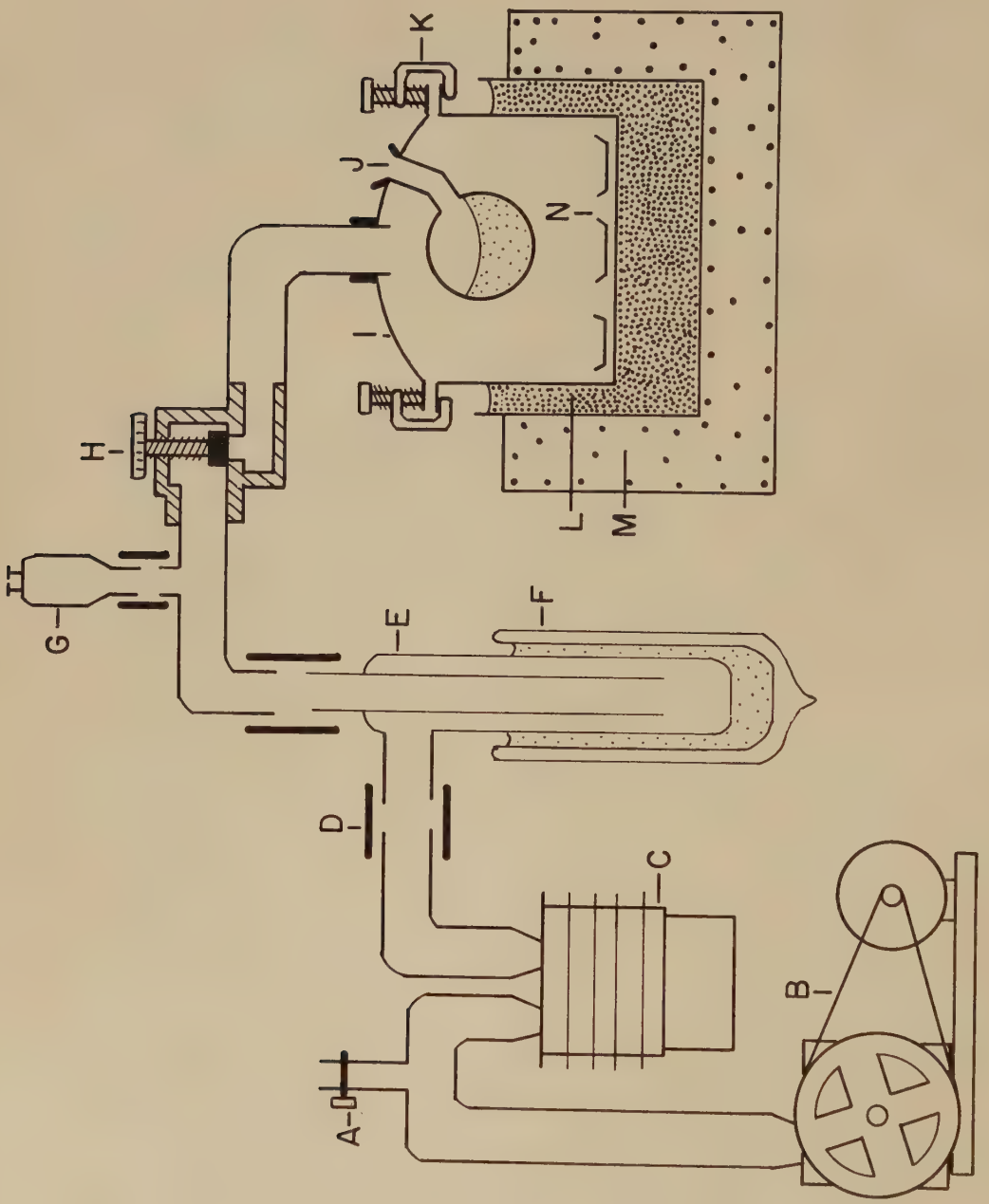

Figure 1

dehydrated tissues were still suitable for chemical as well as morphologic studies. We have, therefore, attempted to provide an apparatus and technique embodying these modifications.

APPARATUS

A diagram of the apparatus is shown in figure 1. Metal or rubber was employed in construction, where possible, in an attempt to limit breakage. Copper tubing, 0.75 inch in diameter, with soldered joints, was used for all connections except those in which high vacuum rubber tubing was employed, as at (D) in figure 1. The entire system was painted with glyptal lacquer to seal small leaks.

The apparatus consisted of two parts, that serving for production and measurement of a high vacuum, and that for dehydration of tissue samples. The high vacuum was attained with a combination of a Welch Duo-Seal pump (B) and an air-cooled oil diffusion pump (C). The pressure inside the system was measured by a Phillips magnetic vacuum gauge, constructed entirely of metal, which had a range of $0.2–250 \times 10^{-4}$ mm Hg. A McLeod gauge was not employed since it is fragile and does not indicate the presence of water vapor or other compressible gases.

According to Simpson ('41), the dehydration must be done while the frozen tissues are maintained at a temperature below $-30°$C. Otherwise, there is a possibility of liquefaction of ice crystals with consequent distortion of cellular morphology and change in chemical constitution. The vapor pressure of ice at $-40°$C. is 9.66×10^{-2} mm Hg. A still lower pressure and temperature was maintained by the pumping system so that the ice crystals sublimed directly into water vapor, and thus were rapidly removed without passing through a liquid state. In practice the high vacuum system was operated at $2 - 5 \times 10^{-4}$ mm Hg, which is well below the limiting pressure. The efficiency ratio was thereby increased.

The dehydrating chamber (I) is an aluminum desiccator whose cover is attached permanently to the vacuum system. After introduction of the tissues, the chamber was sealed in

place by means of a rubber gasket coated with high vacuum silicone grease (Dow-Corning). Four C-clamps (K) secured the chamber to the cover.

The tissues were kept at the optimum temperature of — 40°C. by surrounding the bottom and walls of the chamber with a thick slush (L) of frozen ethyl oxalate (Tech.) in a Fiberglas-insulated container (M). A constant temperature of — 40°C., which is the freezing point of ethyl oxalate, can be maintained for long periods of time since deviations will result only in a change in the proportions of solid to liquid and the establishment of a new equilibrium. The ethyl oxalate

TABLE 1

Relative efficiencies of various drying agents

DRYING AGENT	MG OF WATER PER LITER OF GAS DRIED AT 25°C.
Liquid nitrogen trap	1.6×10^{-23}
Phosphorus pentoxide	2×10^{-5}
Magnesium perchlorate	5×10^{-4}
Calcium chloride	2.5×10^{-1}

(Reprinted from National Research Council, *International Critical Tables, 3:* 385 ['28].)

was initially frozen to a thick slush by the slow addition of powdered "dry ice" with constant stirring. Occasional additions of "dry ice" during the operation of the apparatus were also necessary.

The water vapor formed by sublimation from the tissues must be removed if the dehydration is to proceed. Techniques employed for this purpose by previous investigators incorporated phosphorus pentoxide and other chemical drying agents into the system. In the present apparatus, water vapor was refrozen on the surface of a "cold finger" condenser (J) filled with liquid nitrogen. This is constucted from a round-bottom flask whose neck is sealed through a hole in the chamber cover. Strong ('38) states that liquid nitrogen is

one of the most efficient of known drying agents (see table 1). In our opinion, the lesser efficiency of chemical drying agents compared to liquid nitrogen may be responsible for the longer time (three or more days; Hoerr, '36) required for dehydration by a conventional apparatus. With this modification dehydration is complete in 7 hours.

PROCEDURES AND APPLICATIONS

A basket made of wire mesh is placed in the bottom of a beaker filled with isopentane (Eastman Kodak, Tech.). The beaker is chilled to a temperature of — 195°C. by immersion in a Dewar flask containing liquid nitrogen. The isopentane is continually stirred by a slowly revolving glass paddle.

Pieces of tissue, no larger than 5 mm in diameter, are dropped into the cold isopentane for freezing. About 15 seconds is allowed for this to take place. The wire basket containing the frozen tissues may then be removed and rapidly transferred, either directly to the apparatus or, if more convenient, to an aluminum container packed in "dry ice" at — 72°C. where it can be stored until processed.

The desiccator chamber (I) is placed in the frozen slush of ethyl oxalate which has been prepared in the manner described above, and allowed to cool to — 40°C. Valve (H) is closed, and the system is evacuated as far as the valve by the mechanical (B) and oil diffusion (C) pumps. Wire baskets containing the frozen tissues are then rapidly transferred to the desiccator chamber (I), and a sealing film of silicone grease (Dow-Corning High-Vacuum) is placed on the rubber gasket. The chamber is then clamped in place on the system with the C-clamps (K) and evacuated by opening the valve (H). After about 5 minutes of pumping, the "cold finger" condenser (J) is filled with liquid nitrogen, and a Dewar flask filled with liquid nitrogen is placed around the trap (E) as an additional precaution. The pressure drops to $2 - 5 \times 10^{-4}$ mm Hg as soon as the oil diffusion pump warms up. The

system is then allowed to run for 6 to 8 hours; it requires attention only in the form of occasional additions of liquid nitrogen and "dry ice."

When the drying process is complete, the oil diffusion pump is turned off and allowed to cool. After half an hour valve (H) is closed, the mechanical pump turned off, and the system allowed to return slowly to atmospheric pressure by means of air admitted through pinch-cock (A). All entering air is thereby dried by passage through the trap (E). Valve (H) is then slowly opened. When atmospheric pressure is attained, the chamber is removed and the tissues transferred rapidly to a vacuum desiccator in which they are allowed to return to room temperature and in which they can be stored.

For histological studies the dried tissues may be directly embedded in melted paraffin *in vacuo*. Immersion for 10 to 15 minutes is sufficient. A thermostatically controlled vacuum oven proved to be a useful adjunct in this procedure, and was used also as a vacuum desiccator for storage at room temperature. Blocks of tissue can then be prepared for sectioning.

Special techniques are necessary for stretching and handling cut sections, since floating them on water is obviously contraindicated. The method employed in our laboratory was to cut single sections, and after each section a soft camel's hair brush was used to paint a thick film of melted paraffin on the exposed surface of the block. A rigid backing was thus provided for the next section to be cut, but there was no increase made in the thickness of the tissue section. Sections 7 μ thick can be cut without crumpling. These sections, reinforced by their thick facing of paraffin, may be handled by forceps or spatula. They are placed, cut surface down, on an albumenized slide and secured by gentle pressure. The slide is then warmed to an extent just sufficient to melt the paraffin and plunged into petroleum ether in order to dissolve the paraffin.

The tissue on the slide is now ready to be treated with reagents for histochemical purposes, or may be denatured, as by placing in absolute alcohol for 24 hours, before applying

routine histological stains. In a procedure now under study, sections are mounted in clarite immediately after removal of the paraffin in an attempt to provide permanent but unfixed preparations for phase microscopy.

A series of experiments were carried out in order to determine the efficiency of the apparatus with respect to removal of water from tissues. The wet weights of various tissues of rats were measured upon removal from the animals. The tissues were then subjected to the freezing-drying procedure

TABLE 2

Per cent of water content of various tissues removable by the freezing-drying technique described

ORGAN	WET WEIGHT MG	LOSS OF WEIGHT (MG) AFTER FREEZING-DRYING	LOSS OF WEIGHT (MG) AFTER FURTHER DRYING IN VACUO, 50°C. TO CONSTANT WEIGHT	PER CENT OF TOTAL LOSS OF WEIGHT DURING FREEZING-DRYING
Liver	64.6	40.2	40.3	99.8
Kidney	36.6	25.7	26.0	98.9
Heart	29.9	21.2	21.4	99.1
Intestine	88.0	64.7	65.6	98.7
Lung	28.8	21.1	21.2	99.5
Spleen	40.4	29.0	29.2	99.3
Brain	89.2	65.9	66.4	99.3

described above and allowed to dry for 7 hours. After removal from the apparatus and warming to room temperature, the loss of weight in these tissues was determined. This loss was assumed to be the weight of water removed by the freezing-drying procedure.

To determine the total water content, the tissue samples were then dried in a vacuum oven at 50°C. until a constant weight was obtained. The results for a representative series of tissues (table 2) showed that the efficiency of the apparatus in removing water over a period of 7 hours was 98.5 to

100%, depending on the tissue concerned. Preliminary histological studies indicated that the quality of fixation produced is equal to that of other techniques previously described.

SUMMARY

The freezing-drying method (Altmann, 1890; Gersh, '32) has found wide applicability in the fields of histology and histochemistry. The modification of this method, as described in this article was devised with the following aims in mind: (1) an apparatus made of metal instead of the more fragile glass, (2) simplification and standardization of procedures so that the expense of the apparatus would be decreased and the time of operation would be reduced, and (3) dehydrated tissues prepared so that they would be suitable for chemical as well as for morphologic studies.

It was found that dehydration could be completed in 7 hours, as compared with three or more days required with other apparatus. The efficiency of this technique in removing water from tissues was 98.5 to 100%, depending on the type of tissue under study. Chemical and morphologic changes in the dehydrated tissues therefore appear to be reduced in degree.

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